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PRINCIPAL INVESTIGATOR: Marty W. Mayo, Ph.D.
Carl Creutz, Ph.D.

CONTRACTING ORGANIZATION: University of Virginia
Charlottesville, VA 22904

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Marty W. Mayo, Ph.D.
Carl Creutz, Ph.D.

Email: mwm3y@virginia.edu

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13. SUPPLEMENTARY NOTES

14. ABSTRACT The purpose of our studies is to elucidate how Copine-I antagonizes NF-.B transcription. Nuclear factor-.B (NF-.B) is a dynamic transcription factor that regulates important biological processes involved in cancer initiation and progression. Identifying regulators that control the half-life of NF-.B is important to understanding molecular processes that control the duration of transcriptional responses. In this study we identify Copine-I, a calcium phospholipid-binding protein, as a novel repressor that physically interacts with p65 to inhibit NF-.B transcription. Knockdown of Copine-I by siRNA increases tumor necrosis factor a-stimulated NF-.B transcription, while Copine-I expression blocks endogenous transcription. Copine-I abolishes NF-.B transcription by inducing endoprotease processing of the N-terminus of p65, a process antagonized by I.Ba. Copine-I stimulates endoproteolysis of p65 within a conserved region that is required for base-specific contact with DNA. p65 proteins lacking the N-terminus fail to bind to DNA and act as dominant-negative molecules that inhibit NF-.B transcription. Our work provides evidence that Copine-I regulates the half-life of NF-.B transcriptional responses through a novel mechanism that involves endoproteolysis of the p65 protein. Copine has significance as a potential biomarker for therapeutic intervention in prostate cancer.

15. SUBJECT TERMS Prostate cancer, NF-kappa B, transcription, cell survival, Copine-I, ubiquitin -dependent degradation, apoptosis, chromatin, acetylation, phosphorylation, proto-oncoprotein, tumor suppressor protein, extracellular matrix, tumor necrosis factor alpha, endoproteolysis

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Introduction:

Androgen-ablation is initially an effective form of therapy for prostate cancer (PCa). However, some patients suffering from late-stage PCa often display a recurrence of the disease. One of the intrinsic properties of the recurrent androgen-independent PCa is an exquisite resistance to apoptosis. The androgen-independent PCa cells exhibit resistance to apoptotic cues mediated by death receptor ligands, loss of cellular attachment, as well as chemotherapy-induced cell death (1). In addition to losing functional androgen-receptor signaling pathways, many androgen-independent PCa cells display elevated NF-κB transcriptional activity. In most forms of cancer there are multiple avenues by which carcinomas arise. However, several laboratories have shown that NF-κB activity is dysregulated not only in PCa cells, but that the activation status of this transcription factor may predict the overall responsiveness of the disease to conventional anti-neoplastic therapies (2-5). To understand better how PCa cells become resistant to apoptotic stimuli, it is important to characterize the molecular pathways that control NF-κB-transcription and cell survival. Having previously established that acetylation of p65 at K310 is a prerequisite for full NF-κB transcription (6-8), our laboratory has developed an α-p65(AcK310) antibody and preliminary evidence that this "activation mark" is dysregulated in late-stage human PCa tumors. In order to understand mechanistically why PCa cells display dysregulated acetylated p65, we have identified a novel, naturally occurring, regulator of NF-κB transcription called Copine-I. Copine-I is a calcium-binding protein of previously unknown function. Preliminary data presented in this proposal indicate that Copine-I is an NF-κB regulated gene product that normally functions to control the half-life of the p65 subunit of NF-κB. The overall goal of this proposal is to understand the mechanisms by which Copine-I regulates p65 transcriptional activity and to determine whether Copine-I expression provides a pro-survival signal to androgen-independent PCa cells. To address the hypothesis described above, two aims will be explored. Aim 1 will elucidate the mechanisms by which Copine-I controls p65 turnover. Aim 2 will determine the role of Copine-I as an anti-apoptotic protein. Knowledge obtained from these studies will aid not only in our understanding of how NF-κB becomes dysregulated in PCa cells, but will potentially identify Copine-I as an important regulator of cell survival decisions. This work will potentially impact the way PCa is diagnosed and treated.

Body:

In this the second year of the award, we have made significant progress in all of the original aims of this proposal. Work described below was recently published in Oncogene (9, see Ramsey et al. PDF). Progress during this funding year will be briefly described below.

Progress on Aim 1: Experiments described in Aim 1 were to elucidate the mechanisms by which Copine-I controls p65 turnover. We found that Copine-I is able to inhibit NF-κB transcription mediated by all of the transcriptionally active components of NF-κB, including RelA/p65, RelB and cRel (Fig 1).

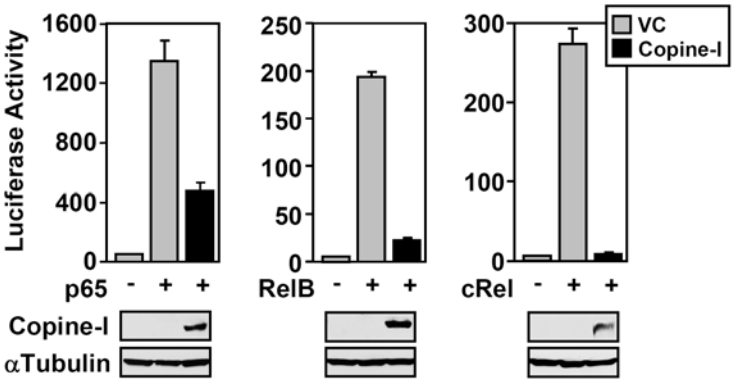


Figure 1: HEK 293T cells were co-transfected with the 3xκB-luciferase reporter and plasmids encoding empty vector (-), p65, RelB, or cRel with and without co-transfection of copine-I expression vector. Cells were harvested after 24 hours. Western blot analysis confirms expression of copine-I. Tubulin expression serves as a protein loading control. All luciferase assays were performed in triplicate and were repeated in three independent experiments. The mean ± standard deviations (SD) are indicated.

This is biologically relevant in PCa cells since a knockdown of Copine-I expression results in an increase in NF-κB gene expression following TNFα addition (Fig 2A). Copine-I interacts with RelA/p65 at an endogenous level (Fig 2B). Copine-I binds to the N-terminus of RelA/p65 (Fig 2C).

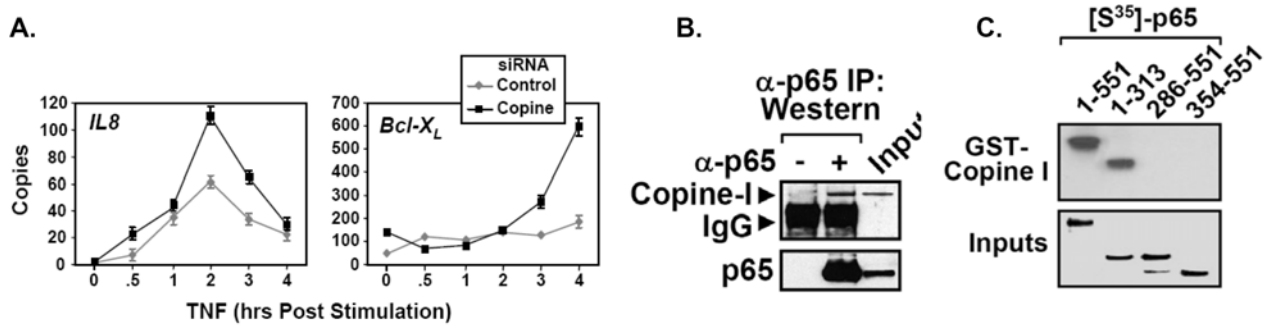


Figure 2: A) Message levels of NF-κB-regulated genes increase in the absence of copine-I. DU145 cells were transfected with copine-I siRNA or control siRNA. QRT-PCR was performed in triplicate with primers specific to *IL-8* or *Bcl-X_L* genes. The relative levels of mRNA expression were normalized to the housekeeping gene *HPRT*. **B)** Endogenous Copine-I interacts with p65. Total input (10%) protein is shown. **C)** *In vitro* translated [^{35}S]-p65 labeled proteins were pulled down with either GST or GST-copine-I. [^{35}S]-labeled p65 proteins were detected by autoradiography. Coomassie stained gels confirm the presence of GST proteins.

To better understand how Copine-I regulates NF-κB transcription, we examined which domains within Copine-I are required to repress RelA/p65 transactivation potential. We found that Copine-I dimerizes through its C2 domain and that the A domain of Copine-I was responsible for inhibiting NF-κB transcriptional activity (Fig 3A). Moreover, we found that membrane localization of Copine-I (Myr-copine) disrupts the ability of Copine-I to repress NF-κB transcription, as measured by quantative real-time PCR (Fig 3B). Copine-I acts directly on the p65 component of NF-κB, rather than disrupting IKK-mediated signaling pathways. Future directions outlined in Aim 1 will elucidate mechanistically how Copine-I inhibits p65 transactivation potential. Although Copine-I was originally proposed to potentiate p65 turnover through ubiquitin-dependent mechanisms, further experimentation clearly indicates that Copine-I functions in a unique manner to block NF-κB transcription. This is evident by the fact that Copine-I expression stabilizes a truncated form of p65, suggesting that rather than potentiating p65 turnover Copine-I promotes stabilization.

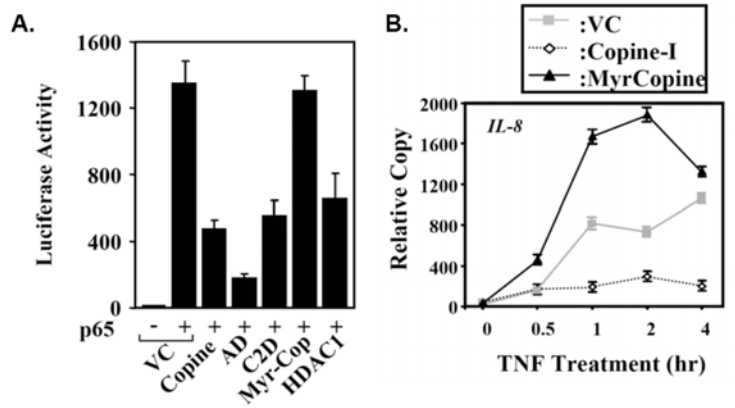


Figure 3: A) Loss of NF-κB transcriptional activity in HEK 293T cells expressing various copine-I expression plasmids. **B)** Localization of copine-I to the membrane increases *IL-8* mRNA expression. HEK 293:VC, HEK 293:copine-I, and HEK 293:myr-copine-I stable cell lines were treated with TNFα for the indicated times. Total RNAs were harvested and QRT-PCR analysis was performed using the *IL-8* primer set.

During our initial analysis, we noticed that Copine-I was controlling the levels of Flag-p65 protein expression. Western blots were performed to address this issue. Cells expressing Copine-I displayed less Flag-p65 protein expression in the soluble RIPA lysate, compared to cells transfected with Flag-p65 alone (Figure 4A). To determine whether the expression of Copine-I was altering the solubility of the Flag-p65, we resuspended cell pellets in 2% SDS and performed Western blots. Although SDS treatment of cell pellets increased our ability to recover Flag-p65, cells co-expressing Copine-I cumulatively displayed less Flag-p65 protein (Figure 4A). Upon further characterization we found that Copine-I was stimulating N-terminal cleavage of Flag-p65, such that the Flag epitope was lost. p65-specific antibodies to either the RHD (α-p65 276) or the C-terminus (α-p65 C-term) identified a 55 kDa immunoreactive peptide in the pellet fraction of cells expressing Copine-I (Figure 4A). These results suggest that Copine-I stimulated endoprotease processing of p65 in the N-terminus resulting in a 55 kDa polypeptide. Importantly, expression of Copine-I alone effectively processed endogenous p65 (Figure 4A). To localize the region within p65 that is cleaved by Copine-I, we created expression plasmids encoding two N-terminal deletion constructs spanning amino acids 31–551 or 51–551. Consistent with data shown in Figure 4A, Copine-I effectively cleaved the full length Flag-p65 protein (Figure 4B). Although less efficient, Copine-I also cleaved the Flag-65(31–551), but was unable to cleave the Flag-p65(51–551) protein. These results suggest that Copine-I cleaves p65 between residues 31 and 51 (Figure

4B). A clustalw alignment of p65 (residues 31–51) with similar domains found in other Rel family members displays a high degree of conservation in amino-acid composition. Copine-I could post-translationally regulate other Rel family members, in addition to p65 including p50, p52, RelB and cRel within the N-terminus of the proteins. These results support the hypothesis that Copine-I is able to direct endoproteolysis of Rel family members within conserved domains required for DNA binding.

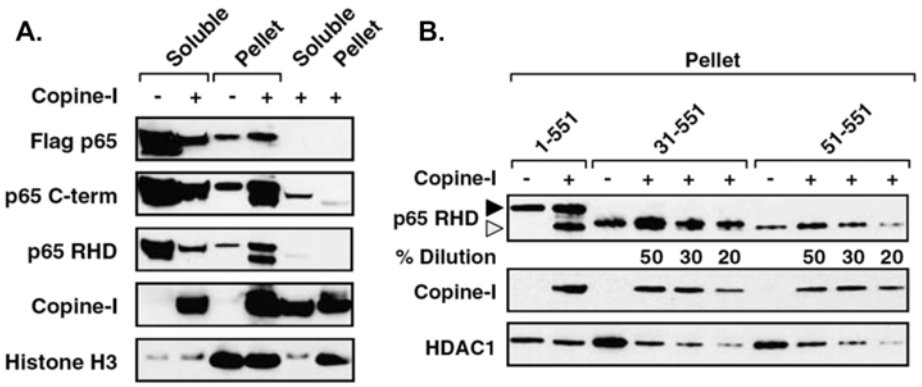


Figure 4: Copine-I cleaves the N-terminus of p65. A) Expression of Copine-I results in endoproteolytic cleavage of Flag p65. HEK 293T cells were transfected with vector control (VC), Copine-I, with or without Flag p65 expression plasmids. Soluble and pellet fractions were collected and western blot analyses performed. The 55 kDa p65 protein was observed using antibodies that detect the Rel homology domain (RHD) or the C terminus of p65. Histone H3 serves as a protein marker for the pellet fractions. **B)** Copine-I induces endoproteolysis of p65 between amino acids 31 and 51. About 5 μl (25 mg total lysate) of p65 (1–551), p65(31–551), p65(51–551) alone and p65 (1–551) + Copine-I were loaded. Decreasing amounts of pellet samples were loaded to visualize the presence of cleaved p65. Percent Dilution 50=10 μg, 30=6 μg, 20=4 μg loaded.

Since the copine-I cleavage site in p65 is located within its DNA-binding domain, we examined whether this truncated form of p65 was functional. Cells were fractionated and nuclear extracts were analysed for the presence of Flag-tagged p65 or p65(51–551). Western blot analysis confirmed that both Flag-p65 and p65(51–551) were localized to the nucleus in the presence of Copine-I expression. Electromobility shift assays (EMSAs) were performed on nuclear extracts to elucidate whether Copine-I altered the ability of the full-length p65 to bind to DNA. Cells expressing Copine-I displayed a significant loss of p65 DNA-binding activity as compared to cells expressing p65 alone (Figure 4A). Consistent with the loss of p65 DNA-binding activity, the p65(51–551) protein was unable to bind to DNA (Figure 4A). Although p65:p65 homodimers are not believed to display the same high affinity for DNA as the p65:p50 heterodimer, both homo- and heterodimers readily form DNA–protein complexes. Consistent with this observation, we detected p65:p65 homodimer and p65:p50 heterodimers complexes, which were confirmed by super-shift analysis using a-Flag and a-p50 antibodies (Figure 4A). As predicted, expression of p65(51–551) failed to stimulate NF-κB transcription. Since a truncated form of p65 would not interact with DNA, we asked whether expression of the p65(51–551) protein could antagonize NF-κB transcription mediated by full-length p65. Expression of p65(51–551) inhibited NF-κB transcription in a dose-dependent manner when co-expressed with full-length wild-type p65 (Figure 4B). To confirm that p65(51–551) was capable of inhibiting NF-κB transcription, we created stable HEK 293 cell lines expressing either VC or the p65(51–551) protein. Cell lines were selected which expressed levels of Flag-p65(51–551) similar to endogenous p65. Consistent with previous observations, cells stably expressing p65(51–551) displayed significantly lower induction of IL-8 transcripts compared to VC cells (Figure 4C). These results indicate that loss of the p65 region comprised of residues 31–51, which corresponds to the Copine-I cleavage site, inhibits NF-κB transcription by deleting residues R33, R35, Y36 and E39, which are critical for base-specific contact of p65 with DNA. Our results are consistent with the hypothesis that Copine-I induces endoproteolysis of p65 and other Rel homology proteins.

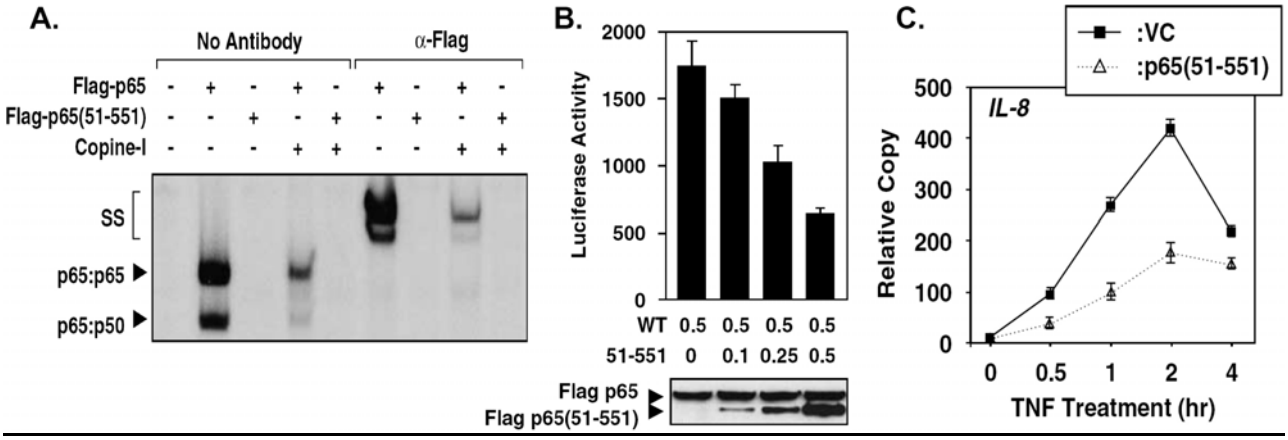


Figure 6: Removal of the DNA-binding domain converts p65 to a transcriptional repressor. A) p65(51–551) does not bind DNA. Electromobility shift assay (EMSA) was performed using nuclear extracts described in DNA–protein complexes were super-shifted (SS) with a-Flag antibody, confirming expression of Flag-p65 proteins. **B)** p65-mediated transcriptional activity is suppressed by p65(51–551) is a dose-dependent manner. Ratios of wild-type full-length p65 (WT) to p65(51–551) plasmids transfected are shown (μg of plasmid per six-well plate). **C)** QRT-PCR demonstrates that HEK 293T cells stably expressing p65(51–551) protein display a lower fold induction of interleukin-1b-8 (IL-8) transcripts, compared to control cells.

Progress on Aim 2: Experiments described in Aim 2 will determine the role of Copine-I as an antiapoptotic protein. Evidence provided thus far indicates that Copine-I negatively regulates NF-κB transcription. NF-κB has been shown to block many different forms of apoptotic stimuli, including those mediated by loss of attachment to the extracellular matrix, a form of apoptosis referred to as anoikis (9). To determine whether Copine-I could sensitize cells to anoikis, we created cell lines stably expressing either Copine-I or Myr-copine. Although control cells displayed increased apoptosis following detachment, cells stably expressing Copine-I were more resistant to anoikis, displaying reduced levels of nucleosome and caspase-3 activity (Figure 7). In contrast, cells stably expressing mislocalized Copine-I (Myr-copine) displayed increased induction of apoptosis following loss of cell attachment. Together these experiments indicate that cells expressing Copine-I cells are more resistant to apoptotic cues despite the fact that these cells displayed reduced NF-κB transcription.

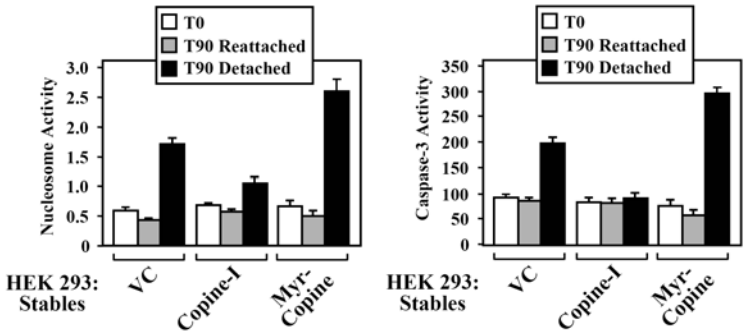


Figure 7: HEK 293:VC, HEK 293:copine-I, and HEK 293:myr-copine-I stable cells lines were either left untreated (T0), or trypsinized and re-plated in serum free media on tissue culture treated dishes (T90 Reattached) or on polyHema coated dishes (T90 Detached). After 90 minutes cells were harvested and apoptosis was measured by nucleosome and Caspase-3 activity assays.

Key Research Accomplishments:

- Human PCa cells display elevated Copine-I protein expression.
- Copine-I is a natural inhibitor of NF-κB transcription.
- Copine-I functions by directly interacting with the p65 subunit of NF-κB.
- Mislocalization of Copine-I to the cell membrane disrupts endogenous Copine activity, which results in increased NF-κB transcriptional activity.
- Copine-I stimulates endoproteolysis of p65 between residues 31–51, which corresponds to residues critical for base-specific contact of p65 with DNA.
- Endoproteolysis of p65 creates a dominant negative protein that acts as a dominant negative inhibitor of NF-κB transcription.
- Although Copine-I blocks NF-κB transcriptional activity, Copine-I expression prevents apoptosis induced in response to cell detachment and TNFα.
- GCN5 acetyltransferase as a novel component of the Cullin/SOCS1/COMMD1 required for polyubiquitination of p65.

Reportable Outcomes:

We have recently published a manuscript describing our researching findings that Copine-I as a novel repressor of NF-κB transcription (9, see Ramsey et al). The Copine project was Catherine Ramsey's main graduate research project and upon the completion of her studies she successfully defended her thesis and was awarded her Ph.D.

Conclusion:

Work from our laboratory has also recently shown that the p65 component allows PCa cells to remain resistant to apoptosis when grown in suspension. Such model systems have been important in demonstrating that resistance to apoptosis following loss of attachment is critical for the development of metastasis of PCa cell to bone (11,12). This work may potentially lead to the development of unique cancer therapies that target NF-κB transcription and components of these signaling pathways. Although we have found that Copine-I is a repressor of NF-κB, the exact mechanism by which Copine-I functions needs further examination. Experimental evidence suggests that Copine-I can modify the p65 component of NF-κB in one of two ways. Chromatin-bound p65 is cleaved into a 55 KD sized protein which no longer contains the N-terminal DNA binding domain. In this way the 55 KD protein acts as a dominant negative protein to unmodified full length p65 protein. However, Copine-I also seems to have another function. Ectopic expression of Copine-I results in posttranslational modification of p65. Interestingly, Copine-I regulates post translational regulation of p65, suggesting that Copine-I may act as a substrate of ubiquitination or be involved in an ubiquitin complex (Figure 8). In support of this we have found that Copine-I interacts with JM1, an adaptor molecule which is an integral member of the Cullin/SOC/COMMD1 complex. This complex has recently been shown to be responsible for degradation of p65 (13). Thus, future experiments will elucidate whether Copine-I antagonizes this ubiquitin complex by the addition of additional posttranslational modifications. In collaboration with Ezra Burstein's laboratory (University of Michigan), we will determine if the 55KD fragment of p65 is resistant to polyubiquitination mediated by the Cullin/SOCS/COMMD1 complex using in vitro and in vivo ubiquitin assays. Since COMD1 interacts with p65 in the first 180 amino acids, we predict that Copine-I dependent processing of p65 will now be resistant to polyubiquitination. In summary, these experiments will determine the molecular mechanisms by which Copine-I functions as a novel regulator of NF-κB transcription and cell survival in PCa cells.

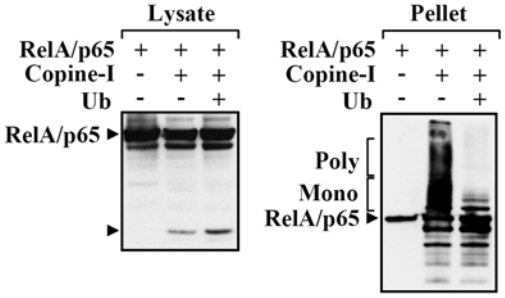
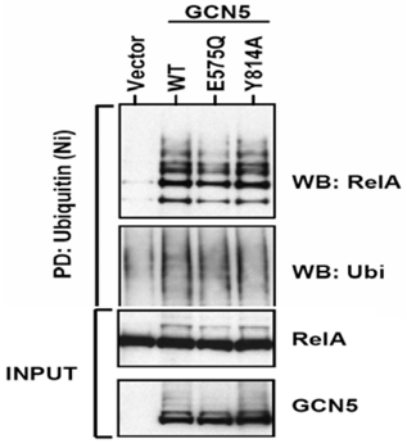


Figure 8: Copine-I mediates posttranslational modification of the p65 subunit. HEK 293 cells were cotransfected with expression plasmids encoding p65, Copine-I, or His tagged ubiquitin. After 24 hrs cells were collected and protein lysates were analyzed by Western.



As an extension of our interest to identify enzyme complexes responsible for polyubiquitination of p65 our laboratory has collaborated with the Burnstein laboratory to prove that GCN5 acetyltransferase as a novel component of the Cullin/SOCS1/COMMD1 (Figure 9, see Mao et al. PDF). Research funds from the DOD Ideal Award were used to support this research. This work resulted in a recent submission which is currently under review.

Figure 9: The HAT activity of GCN5 is not required for p65/RelA ubiquitination and degradation. A) GCN5 promotes RelA ubiquitination despite inactivating mutations in its HAT or Bromo domains. GCN5 wild-type, E575Q (HAT deficient), or Y814A (Bromo domain deficient) were co-transfected with HA-RelA and His6-tagged ubiquitin. Ubiquitinated proteins were subsequently precipitated and the presence of ubiquitinated RelA was determined by immunoblotting (HA). Relative levels of RelA/p65 and GCN5 inputs are shown.

Therefore, in this past funding year research funds from the DOD Idea Development Award PC050549 has lead to one publication (Ramsey et al. Oncogene 2008) and one submitted manuscript under consideration (Mao et al. 2008).

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Appendices:

- Ramsey et al. PDF
- Mao et al. PDF

Supporting Data:

Included in the text.

Oncogene (2008), 1–11

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ORIGINAL ARTICLE

Copine-I represses NF-κB transcription by endoproteolysis of p65

CS Ramsey[1], F Yeung[1], PB Stoddard[1], D Li[1], CE Creutz[2] and MW Mayo[1]

[1]Department of Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA, USA and [2]Department of Pharmacology, University of Virginia, Charlottesville, VA, USA

Nuclear factor-κB (NF-κB) is a dynamic transcription factor that regulates important biological processes involved in cancer initiation and progression. Identifying regulators that control the half-life of NF-κB is important to understanding molecular processes that control the duration of transcriptional responses. In this study we identify copine-I, a calcium phospholipid-binding protein, as a novel repressor that physically interacts with p65 to inhibit NF-κB transcription. Knockdown of copine-I by siRNA increases tumor necrosis factor α-stimulated NF-κB transcription, while copine-I expression blocks endogenous transcription. Copine-I abolishes NF-κB transcription by inducing endoprotease processing of the N-terminus of p65, a process antagonized by IκBα. Copine-I stimulates endoproteolysis of p65 within a conserved region that is required for base-specific contact with DNA. p65 proteins lacking the N-terminus fail to bind to DNA and act as dominant-negative molecules that inhibit NF-κB transcription. Our work provides evidence that copine-I regulates the half-life of NF-κB transcriptional responses through a novel mechanism that involves endoproteolysis of the p65 protein.
Oncogene advance online publication, 21 January 2008; doi:10.1038/sj.onc.1211030

Keywords: NF-κB; copine-I; endoproteolysis; p65; transcription; repressor

Introduction

The transcription factor nuclear factor-κB (NF-κB) controls the expression of many gene products that are important to the development of cancer. NF-κB transcriptional activity is essential for uncontrolled proliferation, enhanced inflammation and initiation and maintenance of epithelial to mesenchymal transition (Huber *et al*., 2004; Karin and Greten, 2005). There are five members in the mammalian NF-κB family, RelA/p65, RelB, cRel, p50/p105 and p52/p100 (Baldwin, 1996). All NF-κB family members contain a Rel homology domain (RHD), through which family members form homodimers and heterodimers. The most studied and predominant form of NF-κB consists of a heterodimeric p65 and p50 complex. NF-κB is sequestered in the cytoplasm by the inhibitory protein, IκB. Upon cellular stimulation, signaling cascades are initiated that converge on the IκB kinase (IKK) complex (Hayden and Ghosh, 2004). This kinase complex phosphorylates IκB, leading to its polyubiquitination by the SCF-type E3 ligase, $E3RS^{IκB/β\text{-}TrCP}$ and subsequent degradation by the 26S proteosome (Karin and Ben Neriah, 2000). The active NF-κB heterodimer is then able to translocate to the nucleus where it replaces the inactive p50 homodimer on chromatin and initiates transcription.

NF-κB stimulates gene transcription by recruiting co-activator complexes that contain histone acetyltransferases (HATs). NF-κB facilitates loading of basal transcription factors and RNA polymerase II onto the NF-κB-regulated promoter. Co-activator complexes contain p300 or CREB-interacting-binding protein (CBP), as well as p300/CBP-associated factor, or members of the SRC kinase family. Our laboratory has shown that acetylation of p65 at lysine 310 by p300 is associated with full transcriptional activity (Yeung *et al*., 2004). To effectively recruit co-activator complexes, the p65 subunit of NF-κB must be post-translationally modified on specific residues. Phosphorylation of p65 at serine 276 or 536 is necessary for exchanging co-repressor complexes for co-activator complexes (Zhong *et al*., 2002; Chen *et al*., 2005; Hoberg *et al*., 2006). Our laboratory has shown that the activation and repression of NF-κB-responsive genes is a dynamic process, with continual exchanges between co-activator and co-repressor complexes (Hoberg *et al*., 2004, 2006). Upon the termination of transcriptional activation, much of NF-κB is removed from the nucleus and sequestered in the cytoplasm by newly synthesized IκBα, which resets the pathway for subsequent reactivation (Chen *et al*., 2001). This mechanism may account for the nuclear export of the NF-κB heterodimer, but it may not account for the termination of NF-κB transcription. Transcriptionally active NF-κB is targeted for proteosomal degradation at the chromatin level (Saccani *et al*., 2004), suggesting that a transcriptionally active subpopulation of NF-κB is degraded rather than exported. Recently, p65 has been shown to be ubiquitinated by the $COMMD1\text{-}ECS^{SOCS1}$ E3 ligase complex (Maine *et al*., 2007). The ability of COMMD1 to interact with p65 in a chromatin-dependent manner

Correspondence: Dr MW Mayo, Department of Biochemistry and Molecular Genetics, University of Virginia, Box 800733, Charlottesville, VA 22908, USA.
E-mail: mwm3y@virginia.edu
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indicates that recruitment of the COMMD1-ECS^{SOCS1} E3 ligase complex most likely controls the turnover of chromatin-bound NF-κB complex (Burstein *et al*., 2005). Therefore, the cell may differentially regulate the fate of the NF-κB complex under the context of transcriptional regulation.

Copines are a family of calcium-dependent lipid-binding proteins that are evolutionally conserved from *Arabidopsis* to *Homo sapiens*. Nine human copine family members have been identified (Tomsig and Creutz, 2002; Maitra *et al*., 2003). Copine-I, -II and -III are ubiquitously expressed, while the other family members are tissue specific. Copines are comprised of two N-terminal C2 domains (C2Ds) and a C-terminal A domain (AD) (Tomsig and Creutz, 2002). The C2Ds of copines contain aspartate residues important for calcium and phospholipid binding (Nalefski and Falke, 1996; Rizo and Sudhof, 1998). The AD shows similarity to proteins that contain the von Willebrand factor (vWF) domain referred to as the vWF A domain (vWA) protein superfamily (Whittaker and Hynes, 2002). Although the majority of vWA-containing proteins in higher eukaryotes is extracellular, the most evolutionally conserved vWA domains are intracellular proteins. Secondary structure predictions and crystal structure data of the vWF indicate that the AD is composed of alternating α-helices and β-strands that adopt a classic α/β Rossmann fold. Similar to many vWA-containing proteins, copine contains a metal ion-dependent adhesion site (MIDAS) motif, which is critical for manganese and magnesium binding (Creutz *et al*., 1998; Tomsig and Creutz, 2000).

Although copine-I was shown to bind several intracellular proteins with diverse biological functions (Tomsig *et al*., 2003), the role of copine-I in regulating biological processes is not well understood. The presence of *cis*-κB-binding elements in the promoter region for *copine-I* provided the initial rationale for investigating whether copine modulated NF-κB transcription in response to tumor necrosis factor-α (TNFα) (Tomsig *et al*., 2004). Work presented here extends these initial studies and provides evidence that copine-I directs N-terminal proteolytic processing of p65 within a conserved region that is required for base-specific contact with DNA. The resulting processed p65 no longer binds DNA, but rather acts as a dominant-negative inhibitor of NF-κB transcription. The ability of copine-I to post-translationally modify p65 is a unique mechanism used by cells to regulate NF-κB transcriptional responses.

Results

Copine-I blocks NF-κB transcriptional activity

To elucidate whether copine-I regulates NF-κB-activity, we performed transient transfections using an NF-κB-responsive luciferase reporter. Prostate cancer cell lines DU145, PC3 and LNCaP were chosen because they are known to display basal and TNFα-inducible NF-κB transcriptional activity (Mayo *et al*., 2002). Expression of copine-I inhibited NF-κB transcription in all cell lines tested following a 4 h stimulation with TNFα (Figure 1a). The loss of NF-κB-mediated transcription was not due to loss of cell viability, because luciferase values were normalized to β-galactosidase activity. The ability of copine-I to block NF-κB transcription was not altered in response to calcium signaling, since the addition of carbachol, a muscarinic receptor agonist, or the calcium ionophore A23187, failed to rescue TNFα-induced NF-κB transcription in cells expressing copine-I (data not shown). Expression of copine-I blocked both TNFα- and interleukin-1β (IL-1β)-induced NF-κB transcriptional activity, indicating that copine-I was inhibiting NF-κB at a common point in the cytokine pathways (Figure 1b). Copine-I also inhibited TNFα-induced activity of the *IL-8* promoter in an NF-κB-dependent manner, in comparison to a mutant *IL-8* promoter lacking functional *cis*-κB DNA-binding elements (Figure 1c). Additional experiments show that cells expressing copine-I displayed reduced NF-κB transcriptional activity mediated by p65, RelB and cRel (Figure 1d). Results shown in Figure 1 are different from those previously published (Tomsig *et al*., 2004). Hemagglutinin (HA)-tagged copine-I used in Figure 1 is expressed in both the cytosol and nucleus (Supplementary Figure 1). However, green fluorescent protein (GFP)-copine-I, used in the previous study, is excluded from the nucleus (JL Tomsig and CE Creutz, unpublished observations). Thus, copine-I localization may explain the difference between our results and those previously published (Tomsig *et al*., 2004). Collectively, our results indicate that copine-I inhibits NF-κB transcriptional activity.

Knockdown of endogenous copine-I increases NF-κB activity

To explore the effect of endogenous copine-I on NF-κB activity, we used small interfering RNA (siRNA) to knockdown copine-I expression. Copine-I siRNA effectively inhibited copine-I mRNA expression in DU145 cells, compared to cells treated with control siRNA (Figure 2a). To confirm the specificity and efficiency of the copine-I siRNA on protein knockdown, we repeated experiments in human embryonic kidney (HEK) 293T cells expressing HA-tagged copine-I or Flag-tagged copine-IV proteins. Copine-I siRNA knocked down HA-copine-I expression without altering Flag-copine-IV protein levels, demonstrating the specificity of the copine-I siRNA (Figure 2b). As a tool to study endogenous copine-I expression, we generated a rabbit polyclonal antibody against a unique peptide sequence in the C-terminal domain of copine-I. The polyclonal antibody recognized endogenous copine-I, demonstrating the specificity of the newly developed α-copine-I antibody (Figure 2c). To elucidate whether endogenous copine-I expression was affecting NF-κB transcription, siRNA was used to knockdown copine-I expression in DU145 cells and quantitative real-time PCR (QRT-PCR) was performed. We chose to evaluate *IL-8* and

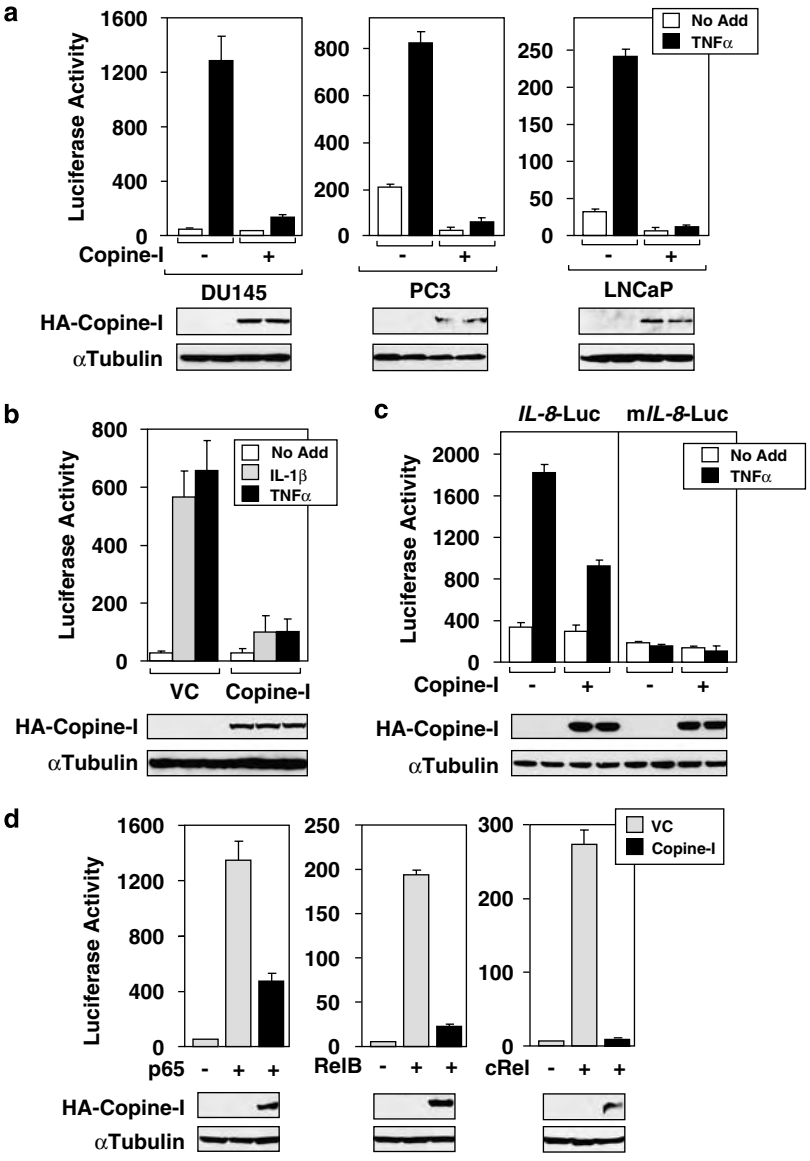

Figure 1 Expression of copine-I inhibits nuclear factor-κB (NF-κB) transcription. (**a**) Prostate cancer cells expressing copine-I display a loss of NF-κB activity. Cells were co-transfected with the NF-κB-responsive 3× κB-luciferase reporter and with vector (−) or copine-I expression plasmid (+). Cells were left untreated (no add) or treated with tumor necrosis factor-α (TNFα) for 4 h. Western blot analyses confirm the expression of HA-copine-I or α-tubulin. All transfections were normalized with cytomegalovirus (CMV)-β-galactosidase activity. Luciferase assays were performed in triplicate and were repeated in three independent experiments. The mean ± standard deviations (s.d.) are indicated. (**b**) Copine-I inhibits TNFα- and interleukin-1β (IL-1β)-stimulated NF-κB activity. (**c**) *IL-8* promoter activity is antagonized by copine-I. HEK 293T cells were co-transfected with the *IL-8*-luciferase reporter or a mutant *IL-8*-luciferase reporter, which lacks functional NF-κB *cis* elements. (**d**) Copine-I blocks NF-κB transcriptional activity mediated by Rel family members.

Bcl-X_L expression because our laboratory has previously shown that these transcripts require NF-κB for transcriptional activity (Hoberg *et al*., 2004). siRNA knockdown of copine-I resulted in increased TNFα-induced *IL-8* and *Bcl-X_L* gene expression, indicating that copine-I inhibits NF-κB transcriptional responses at an endogenous level (Figure 2d).

Copine-I binds to the N-terminus of p65

First, we examined whether copine-I blocked NF-κB nuclear translocation or the transactivation potential of p65. Expression of copine-I neither blocked TNFα-induced degradation of IκBα, nor prevent p65 nuclear translocation (Supplementary Figure 1). Rather, we used Gal4-reporter assays to show that copine-I blocked the transactivation of the full-length Gal4-p65, but not the Gal4-p65 (286–551). Thus, copine-I requires regions within the N-terminus of p65 to inhibit transcription. Next, we examined whether copine-I interacts directly with p65. HA-tagged copine-I interacted with endogenous p65 regardless of whether p65 or epitope-tagged copine-I was immunoprecipitated (Figure 3a). Moreover, endogenous p65 and copine-I protein complexes

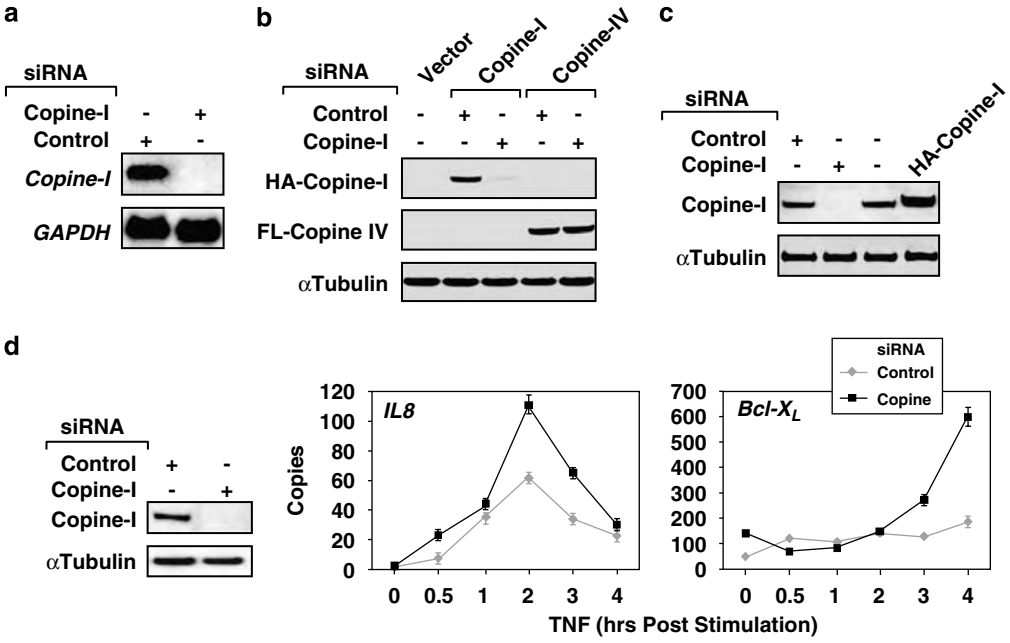

Figure 2 Knockdown of endogenous copine-I leads to increased nuclear factor-κB (NF-κB) transcriptional activity. (**a**) Northern blot analysis for endogenous *copine-I* mRNA expression demonstrates the efficiency of the small interfering RNA (siRNA) copine-I knockdown. (**b**) Western blot using α-HA or α-Flag antibodies confirms the specificity of copine-I siRNA. HEK 293T cells were transfected with copine-I siRNA or control siRNA. Cells were transfected with either copine-I or copine-IV expression vectors. (**c**) The copine-I antibody detects endogenous copine-I. (**d**) DU145 cells transfected with copine-I siRNA display a loss of protein expression. Message levels of NF-κB-regulated genes increase following the knockdown of copine-I. QRT-PCR was performed in triplicate with primers specific to interleukin-8 (*IL-8*) or *Bcl-X_L* genes. The relative level of mRNA expression was normalized to the housekeeping gene *HPRT*.

were observed following immunoprecipitation with α-p65-specific antibodies (Figure 3b). Since results shown in Supplementary Figure 1 suggest that copine-I requires the N-terminus of p65 for transcriptional inhibition, we performed *in vitro* binding assays to localize the interaction. Glutathione *S*-transferase (GST)-copine-I interacted with both the full-length and p65(1–313) proteins, but not with the C-terminal p65 proteins (Figure 3c). In reciprocal experiments, *in vitro* transcribed and translated copine-I interacted with recombinant GST-p65 in the N-terminal region of p65 (Figure 3d). Taken together, these results show that copine-I binds to the N-terminus of p65 between residues 1–286 (Figure 3e). This region of p65 overlaps with the RHD, which contains important functional domains required for DNA binding, IκBα interaction and protein dimerization with other Rel family members (Baldwin, 1996).

Functional characterization of copine-I domains

The structure of copine-I consists of two calcium- and phospholipid-binding C2Ds followed by a vWF A domain, which contains a metal-binding MIDAS motif (Figure 4a). Several mammalian copine-I expression plasmids were generated, including full-length protein, the C2D, the AD and myristilate-tagged full-length copine-I construct (Figure 4a). The Myr-copine construct was designed to artificially mimic cytoplasmic membrane localization of copine-I. Western blot analysis of HEK 293T cells expressing the epitope-tagged constructs confirms protein expression (Figure 4b). Surprisingly, all of the copine-I expression constructs effectively inhibited p65-stimulated NF-κB transcriptional activity except for the Myr-copine-I (Figure 4c). The copine-I AD was the most effective inhibitor of p65 transcription. This result is consistent with the vWA being the functional domain of the copine-I protein (Creutz *et al*., 1998). The inability of the Myr-copine-I construct to block NF-κB-responsive luciferase reporter suggests that membrane localization of copine-I disrupts the ability of copine-I to inhibit NF-κB activity. This hypothesis is supported by the observation that Myr-copine-I localizes the protein to the cytoplasmic membrane (Figure 4e, center panel). The ability of the C2D to inhibit p65 transcriptional activity was unexpected (Figure 4c). C2Ds may function as dimerization domains in the presence of calcium (Tomsig *et al*., 2003). This published result supports our observation that copine-I functions as a dimer where the C2D recruits endogenous copine proteins.

To elucidate whether the C2D binds to full-length copine-I, we performed *in vitro* binding assays. GST-copine-I effectively isolated both full-length and the C2 domain (Figure 4d). However, the GST-copine-I interacted weakly with the AD. The interactions with the full-length GST-copine-I were specific, because neither full-length copine-I nor the truncated proteins bind to GST alone. Our results suggest that copine-I interacts

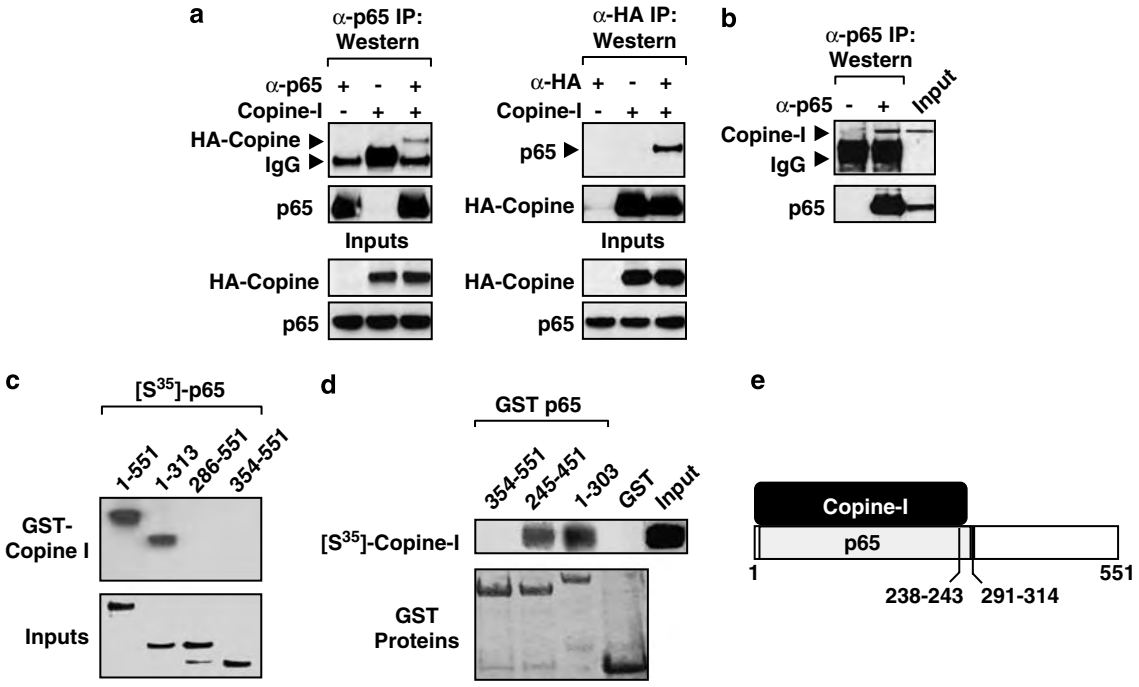

Figure 3 Copine-I interacts with the N-terminus of p65. (**a** and **b**) Copine-I and p65 physically interacts with one another. (**a**) HEK 293T cells were transfected with HA-copine-I expression plasmid (+) or vector control (VC) (−). Cells lysates were immunoprecipitated using α-p65 (+) antibody or normal rabbit immunoglobulin G (IgG) (−), immunoprecipitates were resolved by SDS–polyacrylamide gel electrophoresis (PAGE), transferred, and blots were analyzed for HA-copine-I protein. Reverse immunoprecipitations were performed using αHA (+) or control α-Flag (−) antibodies. Western blot analyses detect endogenous p65 or HA-copine expression in inputs. (**b**) Endogenous copine-I interacts with p65. Extracts were immunoprecipitated using rabbit polyclonal α-p65 (+). Normal rabbit IgG served as a negative antibody control (−). Total input (10%) protein is shown. (**c**) *In vitro* translated [^{35}S]-p65 labeled proteins were pulled down with either glutathione *S*-transferase (GST) or GST-copine-I. [^{35}S]-labeled p65 proteins were detected by autoradiography. Coomassie-stained gels confirm the presence of GST proteins. (**d**) *In vitro* translated [^{35}S]-labeled copine-I proteins interact with GST-p65 proteins. (**e**) Illustration showing the interaction domain between copine-I and p65. The Rel homology domain (RHD) is highlighted in gray and the two IκBα interacting domains are shown (238–243 and 291–314).

with itself through the C2 domain. This may explain how Myr-copine-I might act to relocalize endogenous copines and other components to the cellular membrane.

To determine whether copine-I functions as a natural inhibitor of NF-κB transcriptional responses, we created stable HEK 293 cells expressing vector control (VC), full-length copine-I or Myr-copine-I (Figure 4e). The myristilation sequence effectively localizes copine-I to the cytoplasmic membrane, as confirmed by membrane fractionation assays and western blotting for the Flag epitope (Figure 4e). RNAs were isolated from stable cell lines following the addition of TNFα, and QRT-PCR was performed. HEK 293:VC cells displayed normal induction of *IL-8* gene expression in response to TNFα (Figure 4e). In contrast, HEK 293:copine-I cells failed to display significant levels of *IL-8* transcripts following TNFα stimulation. Surprisingly, HEK 293:Myr-copine cells were more responsive to TNFα-induced *IL-8* gene expression than control cells. Similar patterns of TNFα-induced gene expression were observed for *c-IAP-2* and *Bcl-X_L* using the HEK stable cell lines (data not shown). These results provide evidence that copine-I is an important regulator of endogenous NF-κB transcriptional activity in response to TNFα.

Copine-I cleaves the N-terminus of p65

During our initial analysis, we noticed that copine-I was controlling the levels of Flag-p65 protein expression. Western blots were performed to address this issue. Cells expressing copine-I displayed less Flag-p65 protein expression in the soluble RIPA lysate, compared to cells transfected with Flag-p65 alone (Figure 5a). To determine whether the expression of copine-I was altering the solubility of the Flag-p65, we resuspended cell pellets in 2% SDS and performed western blots. Although SDS treatment of cell pellets increased our ability to recover Flag-p65, cells co-expressing copine-I cumulatively displayed less Flag-p65 protein (Figure 5a). At first we thought that this was due to protein turnover, however, upon further characterization we found that copine-I was stimulating N-terminal cleavage of Flag-p65, such that the Flag epitope was lost. p65-specific antibodies to either the RHD (α-p65 276) or the C-terminus (α-p65 C-term) identified a 55 kDa immunoreactive peptide in the pellet fraction of cells expressing copine-I (Figure 5a). These results suggest that copine-I stimulated endoprotease processing of p65 in the N-terminus resulting in a 55 kDa polypeptide. Importantly, expression of copine-I alone effectively processed endogenous p65 (Figure 5a).

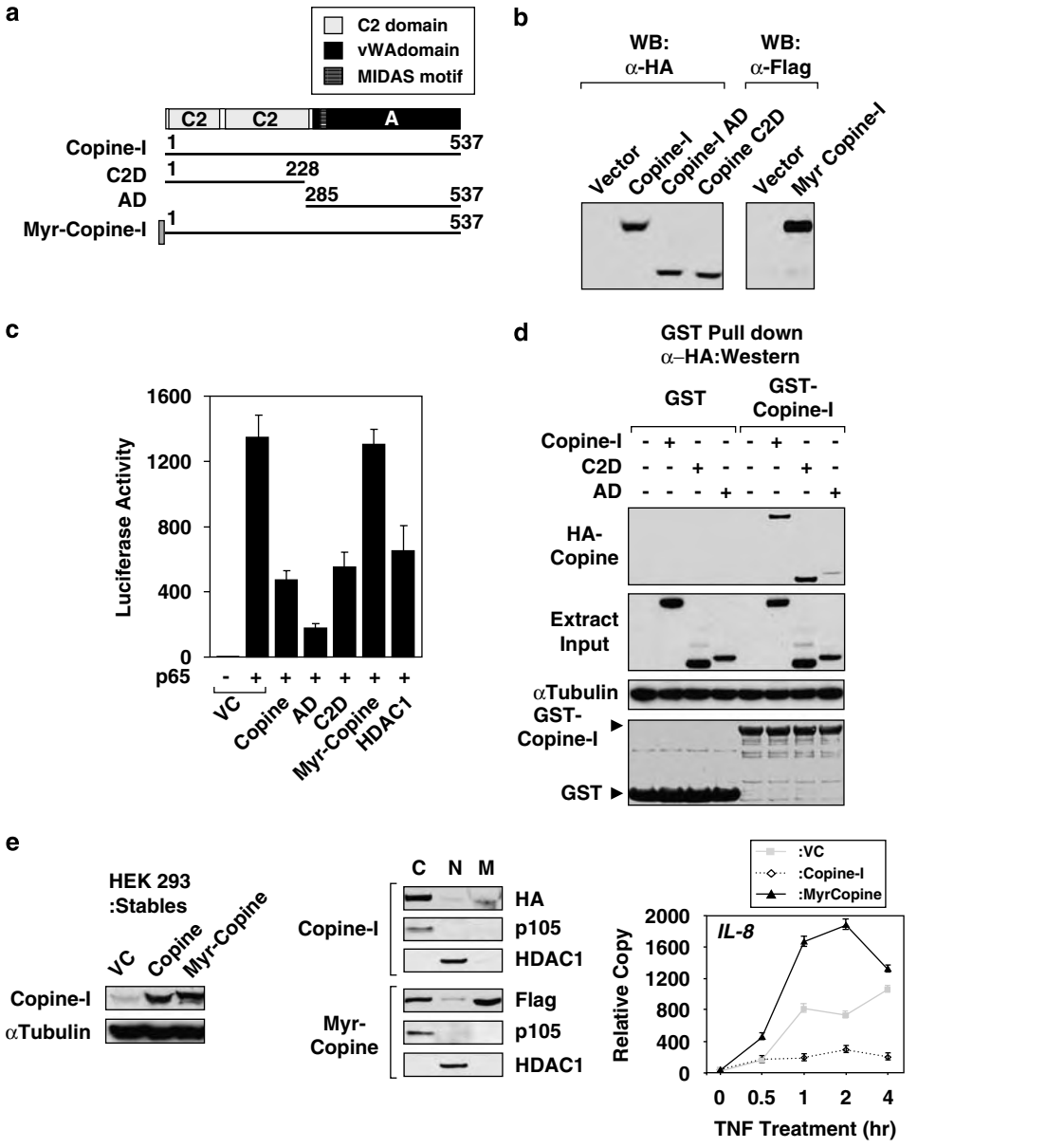

Figure 4 Membrane-associated copine-I no longer represses nuclear factor-κB (NF-κB) transcription. (**a**) Illustration of copine-I protein domains and expression plasmids. (**b**) Western blot analysis of HEK 293T cell extracts displays expression of various copine-I proteins. (**c**) Loss of NF-κB transcriptional activity in HEK 293T cells expressing various copine-I expression plasmids. (**d**) Full-length copine-I interacts with the copine-I C2D. HEK 293T cells were transfected with full-length, C2D or copine-I AD. Whole cell lysates were pulled down overnight with glutathione *S*-transferase (GST)-copine-I. Protein expression in lysates and loading of GST proteins are shown. (**e**) Localization of copine-I to the membrane increases interleukin-1β-8 (*IL-8*) mRNA expression. HEK 293:VC, HEK 293:copine-I and HEK 293:myr-copine-I stable cell lines were treated with tumor necrosis factor-α (TNFα) for the indicated times. Total RNAs were harvested and QRT-PCR was performed using the *IL-8* primer set. Western blot confirms the expression of copine-I and myr-copine-I proteins in stable cell lines. Separation of cell lysates into cytosolic (C), nuclear (N) and membrane (M) fractions demonstrates the localization of myr-copine-I from the cytosol to the membrane. HDAC1 and p105 serve as nuclear and cytoplasmic loading controls.

To understand whether N-terminal cleavage of p65 by copine-I is associated with inhibiting p65 function, we examined whether Myr-copine-I was deficient at endoproteolysis of p65. As observed before, copine-I effectively stimulated endoproteolysis of p65, however, cells expressing Myr-copine-I were less efficient (Figure 5b). Copine-I interacts with p65 across the RHD, which includes one of the two known IκBα-interacting domains (238–243, Figure 3e; Jacobs and Harrison, 1998). Because of this, we investigated whether expression of IκBα could rescue Flag-p65 from endoproteolytic cleavage. Expression of IκBα effectively blocked the ability of copine-I to induce cleavage of p65 (Figure 5c). To localize the region within p65 that is cleaved by copine-I, we created expression plasmids encoding two N-terminal deletion constructs spanning

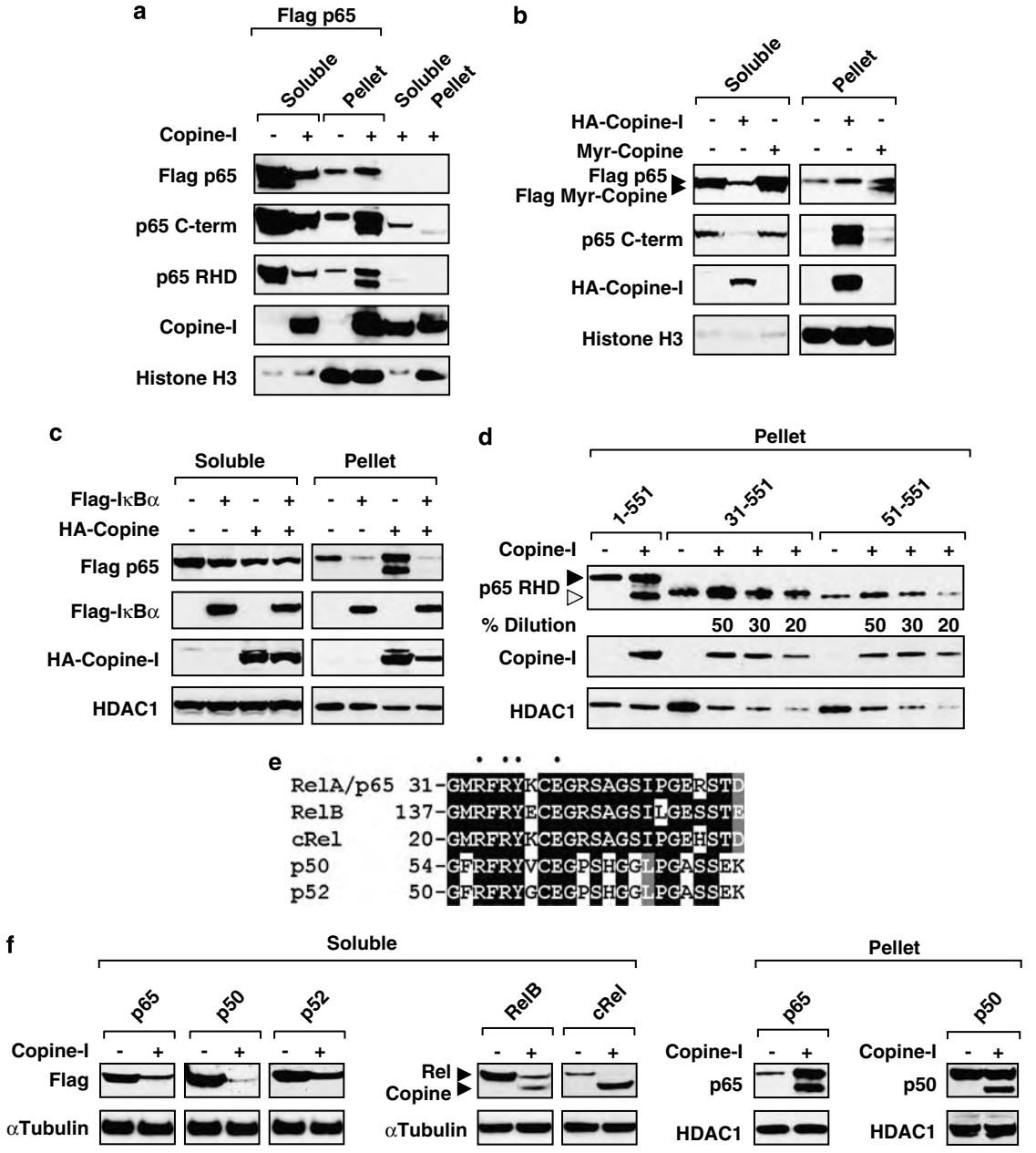

Figure 5 Copine-I cleaves the N-terminus of p65. (**a**) Expression of copine-I results in endoproteolytic cleavage of Flag p65. HEK 293T cells were transfected with vector control (VC) (−), copine-I (+), with or without Flag p65 expression plasmids. Soluble and pellet fractions were collected and western blot analyses performed. The 55 kDa p65 protein was observed using antibodies that detect the Rel homology domain (RHD) or the C terminus of p65. Histone H3 serves as a protein marker for the pellet fractions. (**b**) Myr-copine-I is unable to induce cleavage of Flag p65, as detected by western analysis. (**c**) IκBα protects Flag p65 from copine-I-directed cleavage. HDAC1 serves as a loading control. (**d**) Copine-I induces endoproteolysis of p65 between amino acids 31 and 51. About 5 μl (25 μg total lysate) of p65 (1–551), p65(31–551), p65(51–551) alone and p65 (1–551) + copine-I were loaded. Decreasing amounts of pellet samples were loaded to visualize the presence of cleaved p65. Percent Dilution 50 = 10 μg, 30 = 6 μg, 20 = 4 μg loaded. (**e**) A clustalw alignment of the DNA-binding regions within p65, RelB, cRel, p50 and p52 is shown. p65 residues (R33, R35, Y36 and E39) that interact with DNA are indicated. (**f**) As detected in cell pellet, copine-I causes N-terminal proteolysis of the Rel family members.

amino acids 31–551 or 51–551. Consistent with data shown in Figure 5a, copine-I effectively cleaved the full-length Flag-p65 protein (Figure 5d). Although less efficient, copine-I also cleaved the Flag-65(31–551), but was unable to cleave the Flag-p65(51–551) protein. These results suggest that copine-I cleaves p65 between residues 31 and 51 (Figure 5d). A clustalw alignment of p65 (residues 31–51) with similar domains found in other Rel family members displays a high degree of conservation in amino-acid composition (Figure 5e). This is not surprising because crystal structure analysis of the p65:50 heterodimer reveals that four of the five p65 amino-acid residues and five of the six p50 amino-acid residues located within this region are required for

base-specific contacts with the κB DNA-binding element (Chen FE *et al*., 1998). Next, we evaluated whether copine-I could post-translationally regulate other Rel family members. Copine-I effectively cleaved p65, p50, p52, RelB and cRel within the N-terminus of the proteins (Figure 5f). Similar to p65, cleavage of p50 fragments was relocalized to the pellet fraction, as determined using α-p50-specific antibodies, which detect the C-terminus of the protein (Figure 5f). These results support the hypothesis that copine-I is able to direct endoproteolysis of Rel family members within conserved domains required for DNA binding.

Loss of the DNA-binding domain converts p65 to a transcriptional repressor

Since the copine-I cleavage site in p65 is located within its DNA-binding domain, we examined whether this truncated form of p65 was functional. Cells were fractionated and nuclear extracts were analysed for the presence of Flag-tagged p65 or p65(51–551). Western blot analysis confirmed that both Flag-p65 and p65(51–551) were localized to the nucleus in the presence of copine-I expression (Figure 6a). Electromobility shift assays (EMSAs) were performed on nuclear extracts to elucidate whether copine-I altered the ability of the full-length p65 to bind to DNA. Cells expressing copine-I displayed a significant loss of p65 DNA-binding activity as compared to cells expressing p65 alone (Figure 6b). Consistent with the loss of p65 DNA-binding activity, the p65(51–551) protein was unable to bind to DNA (Figure 6b). Although p65:p65 homodimers are not believed to display the same high affinity for DNA as the p65:p50 heterodimer, both homo- and heterodimers readily form DNA–protein complexes (Chen YQ *et al*., 1998; Phelps *et al*., 2000). Consistent with this observation, we detected p65:p65 homodimer and p65:p50 heterodimers complexes, which were confirmed by super-shift analysis using α-Flag and α-p50 antibodies (Figure 6b; data not shown). As predicted, expression of p65(51–551) failed to stimulate NF-κB transcription (Figure 6c). Since a truncated form of p65 would not interact with DNA, we asked whether expression of the p65(51–551) protein could antagonize NF-κB transcription mediated by full-length p65. Expression of p65(51–551) inhibited NF-κB transcription in a dose-dependent manner when co-expressed with full-length wild-type p65 (Figure 6d). To confirm that p65(51–551) was capable of inhibiting NF-κB transcription, we created stable HEK 293 cell lines expressing either VC or the p65(51–551) protein. Cell lines were selected which expressed levels of

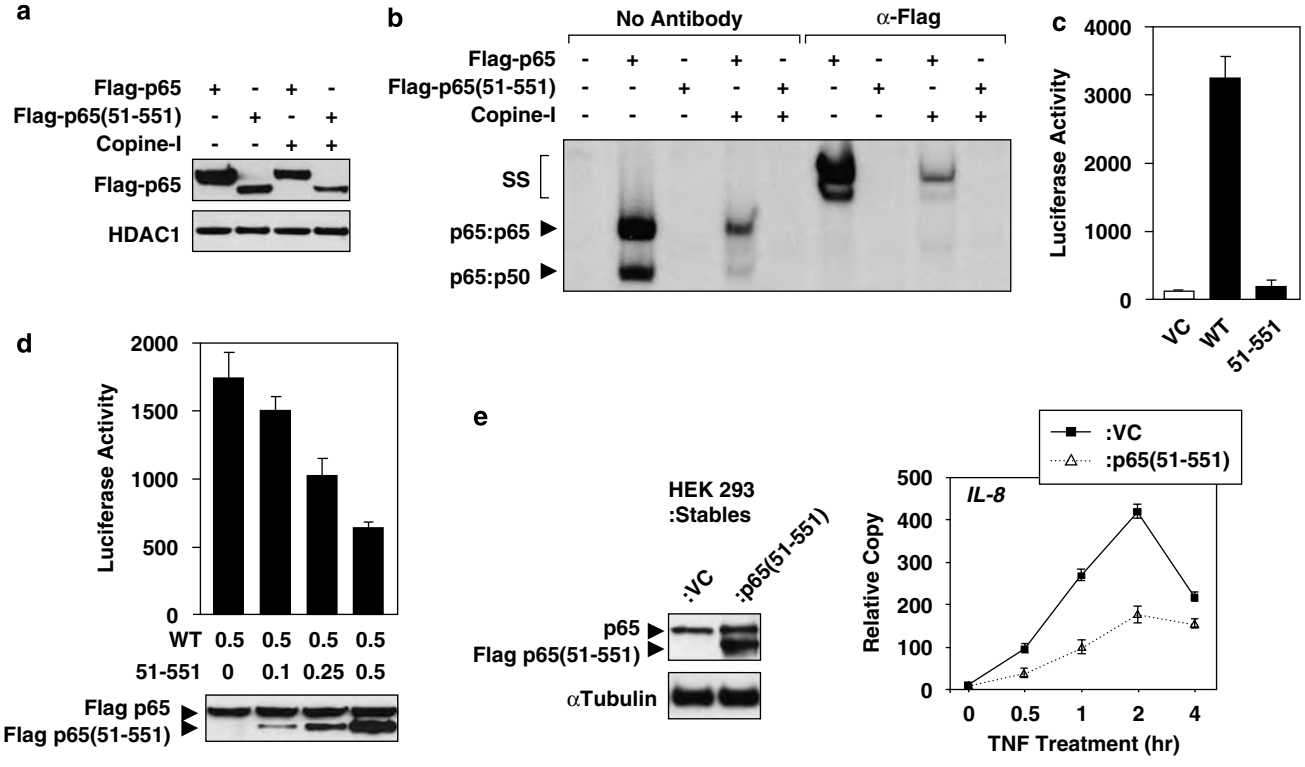

Figure 6 Removal of the DNA-binding domain converts p65 to a transcriptional repressor. (**a**) p65(51–551) is expressed in nuclear extracts. (**b**) p65(51–551) does not bind DNA. Electromobility shift assay (EMSA) was performed using nuclear extracts described in (**a**) DNA–protein complexes were super-shifted (SS) with α-Flag antibody, confirming expression of Flag-p65 proteins. (**c**) p65(51–551) is unable to stimulate nuclear factor-κB (NF-κB) transcription. (**d**) p65-Mediated transcriptional activity is suppressed by p65(51–551) is a dose-dependent manner. Ratios of wild-type full-length p65 (WT) to p65(51–551) plasmids transfected are shown (μg of plasmid per six-well plate). (**e**) Western blot analysis of extracts from stable HEK 293T cells shows the expression of p65(51–551) or vector control (VC). QRT-PCR demonstrates that HEK 23T:p65(51–551) cells display a lower fold induction of interleukin-1β-8 (*IL-8*) transcripts, compared to HEK 23T:VC cells.

Flag-p65(51–551) similar to endogenous p65 (Figure 6e). Consistent with previous observations shown in Figures 6c and d, cells stably expressing p65(51–551) displayed significantly lower induction of *IL-8* transcripts compared to VC cells. These results indicate that loss of the p65 region comprised of residues 31–51, which corresponds to the copine-I cleavage site, inhibits NF-κB transcription by deleting residues R33, R35, Y36 and E39, which are critical for base-specific contact of p65 with DNA. Our results are consistent with the hypothesis that copine-I induces endoproteolysis of p65, which acts as a repressor of NF-κB transcription.

Discussion

In this study, we examined the molecular mechanisms by which copine-I inhibits NF-κB transcription. We show that copine-I does not disrupt the canonical IKK signaling pathway, but rather inhibits NF-κB transcription in a unique way. Using siRNA knockdown and ectopic copine-I expression, we show that copine-I is a natural inhibitor of NF-κB transcription. Copine-I interacts directly with the p65 component of NF-κB, resulting in cleavage of the N-terminus of p65. Crystal structure analysis of the p65:p50 heterodimer bound to the DNA identified eleven base-specific binding residues shared between p65 and p50 (Chen FE *et al*., 1998). Five of these residues are contained within the p65 protein, R33, R35, Y36, E39 and R187. Importantly, four out of the five residues within p65 are located within the N-terminus across the domain targeted by copine-I. Copine-I cleaves p65 within its base-specific DNA-binding domain, resulting in a form of p65 that can no longer interact with the κB *cis* element. As a result, p65 proteins lacking the N-terminus are transcriptionally inactive and yet they are capable of antagonizing TNFα-stimulated NF-κB transcription. More work is needed to understand how truncated p65 protein acts as a repressor. The RHD of p65 interacts with p50 in a region located between residues 222 and 232 (Ruben *et al*., 1992). Since copine-I cleaves p65 across residues 31–51, the 55 kDa form of p65 still contains its p50-interacting domain. Therefore, the p65(51–551) probably acts as a dominant-negative repressor by competitively inhibiting Rel proteins required for DNA binding and NF-κB transcription. This would explain why expression of p65(51–551) effectively inhibits TNFα-induced NF-κB transcription.

Expression of IκBα blocked N-terminal cleavage of p65 by copine-I. These results suggest that protein–protein interactions between IκBα and p65 may prevent copine-I from binding to p65. This is supported by the observation that copine-I interacts with p65 between residues 1 and 286. Since IκBα binds to p65 across two domains (238–243 and 291–314) (Jacobs and Harrison, 1998), expression of IκBα probably prevents copine-I from binding to p65 through steric hindrance. However, we cannot rule out the possibility that cytosolic retention of p65 by IκBα aided the mechanisms protecting p65 from copine-I. The expression of copine-I does not alter NF-κB signaling pathways that involve TNFα-stimulated IKK activation and IκBα degradation (Supplementary Figure 1). However, we did not examine whether copine-I interacted directly with IκB family members. Copine-I was shown to preferentially bind proteins containing coiled-coil and ankyrin-repeat domains (Tomsig *et al*., 2003). In *Caenorhabditis elegans*, copine is require for spermatogenesis, and physically interacts with IκB-1, the ortholog of human atypical IκB molecule, Bcl-3 (Li *et al*., 2004). Thus, copine is evolutionarily conserved and has been shown by other laboratories to bind to the IκB-family members.

Post-translational modification of transcription factors is a common mechanism to regulate the transcriptional response. Polyubiquitination of p65 has been shown to occur at the chromatin level and is believed to be the rate-limiting step in fine-tuning the duration of NF-κB transcription (Saccani *et al*., 2004). Recently, p65 was shown to be degraded by an E3 ligase complex, ECS^{SOCS1}, which is recruited to p65 by COMMD1 (Maine *et al*., 2007). COMMD1 is the rate-limiting factor that recruits elongins, Cul2 and SOCS1 in a TNFα-dependent manner to facilitate p65 turnover. Thus, COMMD1-mediated polyubiquitination of p65 regulates the duration of NF-κB transcription by controlling the half-life of the p65 protein. Our laboratory has previously shown that phosphorylation at S536 and acetylation at K310 are required for chromatin-dependent modifications that result in NF-κB-directed transcription (Hoberg *et al*., 2006). These two chromatin-associated post-translational marks are observed with transcriptionally active and short-lived p65. In stark contrast to COMMD1, we found that copine-I-mediated endoproteolysis of p65 results in a 55 kDa polypeptide that is resistant to degradation. Western blot analysis demonstrates that as a consequence, the 55 kDa p65 protein displays constitutive pS536 and AcK310 immunoreactivity (data not shown). This suggests that copine-I alters the fate of p65, such that it is no longer susceptible to polyubiquitination and degradation. Work is underway to determine whether copine-I protects p65 from the COMMD1-ECS^{SOCS1} E3 ligase through endoproteolysis of p65. Although copine-mediated endoproteolysis of p65 inhibits NF-κB transcription, the biological relevance of this mechanism is not fully understood. More work is needed to determine whether copine-I differentially modulates cell survival advantage in response to select pro-apoptotic stimuli. Consistent with this notion, *copine* gene expression is elevated in late-stage breast and gastrointestinal tract cancers (Wilson *et al*., 2002; Huang *et al*., 2003; Kim *et al*., 2005). Thus, microarray analysis of human tumors supports the hypothesis that *copine* expression is associated with poor clinical outcomes.

Data suggest that copine-I itself does not have endoprotease activity, but rather acts as adapter molecule that recruits enzymatic activity required to proteolytically process p65. This may reflect a more

general function for the copine family of proteins in recruiting proteins bound to their ADs to membranes or to protein complexes (Tomsig *et al*., 2003). Immunoprecipitated copine-I, and recombinant GST-copine-I alone or mixed with cell extracts, were both unsuccessful in stimulating proteolysis of [^{35}S]-labeled p65 *in vitro* (data not shown). Moreover, the addition of the cell-permeable serine protease, lysosomal and proteosome inhibitors, failed to overcome proteolysis of p65 following the expression of copine-I (data not shown). Analysis of the Rel family of proteins revealed a conserved glycine-rich repeat GX_3GX_3G located just upstream of the conserved DNA-binding domain (Figure 5e). This is a similar glycine-rich region identified in p105, which is required for proteosomal processing of p105 into the active p50 subunit (Lin and Ghosh, 1996). However, delivery of permeable transmembrane peptides containing this glycine-rich repeat from p65 could not confirm the importance of this domain for copine-I processing of the Rel family of proteins (data not shown).

This is not the first time NF-κB components have been shown to be regulated by endoproteolysis. NF-κB is an important transcription factor that is targeted by serine and cysteine proteases. In response to apoptotic cues, p65 is targeted for site-directed proteolytic digestion by the cysteine proteases caspase-3 and -6 (Levkau *et al*., 1999; Kang *et al*., 2001). Caspase-mediated processing of p65 blocks NF-κB transcription, suggesting that this is a common mechanism by which cells undergoing apoptosis disrupt NF-κB-mediated cell survival mechanisms. p65 has also been reported to be proteolytically digested by serine proteases (Preston *et al*., 2002). PR3 is tissue specifically expressed in neutrophils, and interacts with CD11b/CD18 (β2 integrin), the major adhesion molecule in neutrophils (Kurosawa *et al*., 2000; David *et al*., 2003). All β2 integrins form a functional heterodimer complex with integrin α subunits that recognize ligand binding via the vWA domain (Dickeson and Santoro, 1998). It is the vWA domains of the integrin subunits that show significant homology with the copine-I AD (Whittaker and Hynes, 2002). Therefore, perhaps one of the evolutionarily conserved functions of vWA domains is to associate with protease-containing protein complexes. This hypothesis is supported not only by PR3 interactions with integrins, but also by the ADAM metalloproteases that are known to interact and mediate site-specific cleavage of the vWF protein (Fujikawa *et al*., 2001; David *et al*., 2003). Future work in our laboratory will focus on identify copine-I-interacting proteins associated with N-terminal processing of p65.

Materials and methods

Cell culture, reagents and plasmid constructs

HEK 293, HEK 293T, prostate cancer cells, DU145, PC3 and LNCaP cells were obtained from ATCC (Manassas, VA, USA). Plasmids encoding the 3 × κB-luciferase, Gal4-luciferase, Flag-p65, Gal4, Gal4-p65 (1–551), Gal4-p65 (286–551) and GST-p65 were described previously (Hoberg *et al*., 2006). HA-p65 expression construct was created by subcloning into pCMV-HA (Clontech, Mountain View, CA, USA). GST-p65 (245–451) was generated by subcloning into pGEX-2T (Amersham Biosciences, Piscataway, NJ, USA). p65 plasmids used for *in vitro* transcription and translation (1–551), (1–313), (286–551), (354–551) and HDAC1 expression plasmid, were described previously (Ashburner *et al*., 2001). Flag-p65(31–551) and Flag-p65(51–551) were generated by subcloning into pFLAG-CMV2 (Sigma, St Louis, MO, USA). *IL-8*-luciferase reporter and mutant *IL-8*-luciferase reporter were provided by the Dr R Natarajan, Medical College of Virginia, Richmond, VA, USA. Expression plasmids encoding HA-RelB and HA-cRel were provided by Dr G Natoli, Bellinzona, Switzerland. Flag-IκBα expression construct was provided by Dr D Ballard, Vanderbilt U, Nashville, TN, USA. HA-copine-I, Flag-copine-I, HA-copine-I C2D, HA-copine-I AD expression plasmids and GST-copine-I were created by subcloning sequences from GFP-copine-I (Creutz *et al*., 1998) into pCMV-HA, pFLAG-CMV2 or pGEX-2T. Myristilate-copine-I (myr-copine) was created by subcloning myristilation sequence, MGSSKSKPKSR, in frame 5′ of the Flag epitope in a Flag-copine-I plasmid. Copine-IV cDNA was obtained from ATCC and subcloned into pFLAG-CMV2. HEK 293 cells stably expressing plasmids encoding copine-I, copine-I AD, myristilate-copine-I or p65(51–551) were generated by transfection of expression constructs and selection of positive clones using the puromycin resistance plasmid pBabe-puro (Dr R Weinberg, Whitehead Institute, Cambridge, MA, USA). Copine-I antibody was generated using the unique C-terminal peptide coupled to keyhole limpet hemocyanin antigen. Polyclonal rabbit antibodies are available through Lake Placid Biologicals, Lake Placid, NY, USA (AR-0167).

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Corresponding Author: Dr. Ezra Burstein, M.D.

Corresponding Author's Institution: University of Michigan

First Author: Xicheng Mao

Order of Authors: Xicheng Mao; Nathan Gluck; Gabriel N Maine; Duo Li; Aparna Repaka; Christine M Komarck; Marty W Mayo; Ezra Burstein

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Abstract: The transcription factor NF-κB is a critical regulator of inflammatory and cell survival signals. Proteasomal degradation of NF-κB subunits through a multimeric ubiquitin ligase containing COMMD1 plays an important role in transcriptional termination. We find that RelA phosphorylation accelerates its ubiquitination, a phenomenon that is prevented by IKK deficiency. To uncover a mechanism connecting RelA phosphorylation and ubiquitination, we searched for additional components of the ubiquitin ligase. We report that GCN5, a histone acetyltransferase, associates with COMMD1 and other components of the ligase, promotes RelA ubiquitination, and represses κB-dependent transcription. Interestingly, GCN5 binds preferentially to phosphorylated RelA, an event that is dependent on IKK activity. Like the IKK complex, GCN5 is also required for phosphorylation-dependent degradation, and in this role, the acetyltransferase activity of GCN5 is completely dispensable. This identifies a previously unknown function of GCN5 in the ubiquitination process and provides a link between phosphorylation and ubiquitination of RelA.

GCN5 Links RelA Phosphorylation and Ubiquitination through a COMMD1-Containing Ubiquitin Ligase

Running Title: GCN5 and RelA ubiquitination

Xicheng Mao[1,2], Nathan Gluck[1,2], Gabriel N. Maine[1,2], Duo Li[3], Aparna Repaka[1,2], Christine M. Komarck[1,2], Marty W. Mayo[3], Ezra Burstein[1,2,4,§]

Department of Internal Medicine[1] and Molecular Mechanisms of Disease Program[2], University of Michigan Medical School, Ann Arbor, Michigan, 48109, U.S.A.; Department of Biochemistry and Molecular Genetics[3], University of Virginia, Charlottesville, Virginia, 22908, U.S.A.; Gastroenterology Section at the Ann Arbor VA Medical Center[4], Ann Arbor, Michigan, 48105, U.S.A.

[§]Corresponding Author: Ezra Burstein, M.D., 109 Zina Pitcher Place – SPC 2200, Biomedical Science Research Building, Room 1526, Ann Arbor, MI 48109; Tel: 734-615-1172, Fax: 734-647-7950, Email: ezrab@umich.edu

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SUMMARY

The transcription factor NF-κB is a critical regulator of inflammatory and cell survival signals. Proteasomal degradation of NF-κB subunits through a multimeric ubiquitin ligase containing COMMD1 plays an important role in transcriptional termination. We find that RelA phosphorylation accelerates its ubiquitination, a phenomenon that is prevented by IKK deficiency. To uncover a mechanism connecting RelA phosphorylation and ubiquitination, we searched for additional components of the ubiquitin ligase. We report that GCN5, a histone acetyltransferase, associates with COMMD1 and other components of the ligase, promotes RelA ubiquitination, and represses κB-dependent transcription. Interestingly, GCN5 binds preferentially to phosphorylated RelA, an event that is dependent on IKK activity. Like the IKK complex, GCN5 is also required for phosphorylation-dependent degradation, and in this role, the acetyltransferase activity of GCN5 is completely dispensable. This identifies a previously unknown function of GCN5 in the ubiquitination process and provides a link between phosphorylation and ubiquitination of RelA.

KEYWORDS: NF-κB / RelA / GCN5 / COMMD1 / IKK / Ubiquitination

INTRODUCTION

NF-κB is a dimeric transcription factor formed by members of a highly conserved family of proteins that share a ~ 300 amino acid sequence termed the *R*el *H*omology *D*omain (RHD) (Karin and Lin, 2002). The activity of NF-κB is regulated primarily by cytoplasmic sequestration of the NF-κB dimer. In the canonical NF-κB pathway, this is the result of interactions between the inhibitory IκB proteins and NF-κB complexes (Baeuerle and Baltimore, 1988). Activation of a multimeric kinase known as the IκB Kinase (IKK) complex results in phosphorylation of IκB, which is then ubiquitinated and degradated by the proteasome (Chen et al., 1995; Henkel et al., 1993). IκB degradation enables the translocation of NF-κB complexes to the nucleus where they bind to cognate DNA sequences present in an array of promoters ultimately resulting in induction of gene expression.

Through its effects on transcription, NF-κB participates in a number of biologic processes including innate and adaptive immunity, programmed cell death, transcriptional regulation of viral replication, cell cycle progression, and oncogenesis (Silverman and Maniatis, 2001). Once transcription has occurred, termination of NF-κB activity is mediated by re-synthesis of IκB proteins, which facilitate nuclear export of NF-κB (Arenzana-Seisdedos et al., 1997). However, besides the role of IκB proteins, additional mechanisms for transcriptional regulation are becoming increasingly recognized. These include a number of post-translational modifications of NF-κB subunits such as phosphorylation (Sakurai et al., 1999; Zhong et al., 1998), acetylation (Chen et al., 2001) (Kiernan et al., 2003), prolyl-isomerization (Ryo et al., 2003) and more recently, ubiquitination (Saccani et al., 2004)n, which are critical in the control of NF-κB activity.

*Co*pper *M*etabolism *M*urr1 *D*omain-containing (COMMD) proteins are a group of evolutionarily conserved factors present in a wide range of organisms (Burstein et al., 2005). COMMD1, the prototype member of the family, was identified as a global inhibitor of NF-κB and binds to a conserved domain present in the RHD of all NF-κB subunits (Burstein et al., 2005; Ganesh et al., 2003; Maine et al., 2007). The effects of COMMD1 on NF-κB lead to decreased pro-inflammatory gene expression (Maine et al., 2007) and have been implicated in the role of this factor in controlling the HIV-1 life cycle (Ganesh et al., 2003).

We recently reported that the ability of COMMD1 to inhibit NF-κB is the result of increased ubiquitination and proteasomal degradation of NF-κB subunits (Maine et al., 2007). COMMD1 is part of a multimeric ubiquitin ligase that also contains *E*longins B and C, *C*ul2 and the *S*OCS box protein SOCS1that is capable of ubiquitinating NF-κB subunits, designated as the ECS^{SOCS1} complex (Maine et al., 2007; Ryo et al., 2003). As part of this complex, COMMD1 cooperates with SOCS1 in the binding of the substrate to the ligase through their concurrent interaction with the amino-terminus of RelA.

The identification of the role of COMMD1 in the ubiquitination of RelA did not suggest any potential link between other post-translational modifications of RelA and its ubiquitination. In this regard, work from Lawrence and colleagues implicated the phosphorylation of RelA at its carboxy terminus by IKKα to the subsequent degradation of this NF-κB subunit, although the precise pathway of degradation has not been elucidated yet (Lawrence et al., 2005). Since RelA binding by COMMD1 and SOCS1 is mediated by the amino-terminus of the RHD, our understanding of this ligase did not provide an obvious mechanism by which phosphorylation of RelA at its carboxy-terminus could be coupled to ubiquitination.

In this report, we describe the identification of GCN5, a histone acetyltransferase (HAT), as an additional component of the ubiquitin ligase that targets RelA. GCN5 is well-known as a transcriptional activator through its association with the RNA Pol II holoenzyme and co-activator complexes such as TFTC and STAGA (Thomas and Chiang, 2006). However, we found that GCN5 functions as a repressor of NF-κB mediated transcription by promoting the ubiquitination and degradation of RelA, a function consistent with its interaction with the COMMD1-containing ligase. While COMMD1 and SOCS1 interact with the amino-terminus of RelA, GCN5 interacts primarily with the extreme carboxy-terminus of RelA, particularly with the phosphorylated form of the protein. Interestingly, unlike its role as a transcriptional activator in other contexts which relies on its ability to acetylate histones (Wang et al., 1997), GCN5-promoted RelA ubiquitination is independent of its HAT activity and represents a novel HAT-independent biochemical role for GCN5. Finally, our findings indicate that the ability of GCN5 to bind to phosphorylated RelA would serve to recruit this protein to the ligase and provides a link between IKK-mediated phosphorylation, ubiquitination and subsequent degradation of this protein.

RESULTS

IKK-mediated phosphorylation results in ubiquitination and degradation of RelA

It has been reported that IKK-mediated phosphorylation of RelA promotes its degradation, but the mechanism that mediates this event has not been reported (Lawrence et al., 2005). As shown in Figure 1A, exposure of wild-type mouse embryo fibroblasts (MEFs) to Calyculin A, a potent inhibitor of protein phosphatase 2A, was accompanied by accumulation of phosphorylated RelA and concurrent degradation of the protein (Figure 1A). RelA phosphorylation was readily apparent by mobility changes on SDS-PAGE (top two panels) or by immunoblotting with a phospho-RelA specific antibody (directed to phosphorylated serine 536, third panel).

We examined next whether RelA degradation in this setting could be explained by increased ubiquitination in response to phosphorylation. To that end, cells were briefly exposed to Calyculin A (without enough time to induce significant degradation), and the proteasome inhibitor MG-132 was also added to stabilize ubiquitinated proteins. Endogenous RelA was immunoprecipitated and the recovered material was immunoblotted for ubiquitin to detect ubiquitinated RelA. In order to avoid the potential co-precipitation of another protein that might also be ubiquitinated, the immunoprecipitation was performed after subjecting the cell lysates to denaturation. In this experiment, proteasome inhibition promoted the accumulation of ubiquitinated RelA, particularly when the cells were also treated with Calyculin A (Figure 1B, upper panel). In addition, the accumulation of phosphorylated RelA in response to Calyculin A treatment was more dramatic when the proteasome was also inhibited, as shown by the greater mobility retardation of RelA (Figure 1B, second and third panels), and upon immunoblotting

with phospho-specific antibodies, which demonstrated greater amounts and additional immunoreactive bands (Figure 1B, bottom panel). Altogether, these data indicated that phosphorylated RelA undergoes ubiquitination and proteasomal degradation.

The use of a phosphatase inhibitor to cause the accumulation of phosphorylated RelA could not identify the specific kinase required for its degradation. Since IKKα-mediated phosphorylation has been linked to RelA degradation, we investigated next whether the degradation of RelA in response to Calyculin A treatment was dependent on the IKK complex. To that end, MEFs from wild-type, *Ikkα* $^{-/-}$, or *Ikkβ* $^{-/-}$ embryos were exposed to Calyculin A. Deficiency of the respective IKK subunit was confirmed by immunoblotting (data not shown). As shown in Figure 1C, deficiency of either IKK subunit markedly decreased the degradation of RelA (top two panels) and greatly impaired the accumulation of phosphorylated RelA (third and fourth panels). These results indicated that IKK-mediated phosphorylation is required for RelA degradation in Calyculin-treated cells.

Identification of GCN5 as a COMMD1-associated factor

We have previously performed a screen for COMMD1-associated factors through large-scale purification of overexpressed COMMD1 in fusion with the TAP affinity tag (Burstein et al., 2005). Among the several potential associated factors identified, which included other COMMD proteins, the sample analysis suggested a possible interaction with the histone acetyltransferase GCN5. A unique peptide from the carboxy terminus of GCN5 was identified by LC/MS-MS (Figure 2A). Next, we investigated if this potential interaction could be confirmed through independent co-immunoprecipitation experiments. The ability of overexpressed COMMD1 and GCN5 to co-precipitate was examined first (Figure 2B, left

panels). Precipitation of COMMD1 (expressed in mammalian cells in fusion with the GST affinity tag) resulted in the co-precipitation of GCN5, an event not observed in the GST transfected negative control.

COMMD1 contains a carboxy-terminal area of homology termed the COMM domain which is required for COMMD dimer formation, as well as the interaction of COMMD1 with Cul2, Elongin C and SOCS1 (Burstein et al., 2005; Maine et al., 2007). Therefore, we examined the role of the COMM domain in COMMD1-GCN5 interactions. The amino-terminus of COMMD1 interacted poorly with GCN5, while the COMM domain in isolation was able to co-precipitate GCN5 nearly to the extent of the full-length protein (Figure 2B, right panels).

Finally, we examined whether endogenous COMMD1 and GCN5 could be co-immunoprecipitated (Figure 2C). Given that GCN5 is exclusively nuclear, these experiments were performed with nuclear extracts, where a pool of COMMD1 has been previously observed (Burstein et al., 2005). In addition, cells were also stimulated with TNF, since such treatment has been shown to promote certain COMMD1 interactions, such as its binding to Cul2 (Maine et al., 2007). This experiment demonstrated that endogenous nuclear COMMD1 can co-immunoprecipitate endogenous GCN5, and this interaction is not substantially affected by TNF treatment (Figure 2C). Altogether, we were able to confirm the interaction between COMMD1 and GCN5 suggested by the TAP screen, and this interaction was further mapped to the COMM domain of COMMD1.

GCN5 inhibits NF-κB mediated transcription

We examined next whether the COMMD1-GCN5 interaction was functionally related to the role of COMMD1 in NF-κB mediated transcription. The first approach to this question was

to measure κB-dependent transcription through a κB-containing reporter plasmid (2κB-luciferase). RelA expression induced reporter activation, which was modestly inhibited by COMMD1. GCN5 expression inhibited RelA-mediated transcription, and this effect was more notable upon its concurrent expression with COMMD1 (Figure 2D). Next, we examined the effect of GCN5 deficiency in TNF-mediated activation of endogenous NF-κB responsive genes. Depletion of GCN5 through transient transfection of small interfering RNA (siRNA) resulted in 10% GCN5 expression at the mRNA level (by quantitative real-time RT-PCR). The GCN5-deficient cells showed greater induction of *TNF* and *CX3CL1* mRNA in response to stimulation (Figures 2E and 2F). Similar experiments were performed after stable down-regulation of GCN5 in U2OS cells, which harbor wild-type p53. This was accomplished by introducing a short hairpin RNA (shRNA) expressing cassette *via* lentiviral infection (Figure 2G). Again, GCN5 deficiency resulted in exaggerated NF-κB mediated expression of *ICAM1* and *IL8* after TNF stimulation (Figures 2H and 2I). Altogether, these results demonstrated that contrary to its role as a HAT, GCN5 functions to repress NF-κB mediated transcription.

GCN5 interacts with the extreme carboxy-terminus of RelA

In order to understand the mechanism GCN5-mediated inhibition of NF-κB we examined whether GCN5 could physically interact with RelA, the most abundant of its subunits. Recombinant GCN5 protein fused in its carboxy terminus with GST was prepared in *E. coli* and its ability to bind to *in vitro* translated radiolabeled RelA was examined (Figure 3A). GCN5 bound avidly to RelA, when comparing the recovered material to the offered input. The ability of endogenous GCN5 and RelA to interact was similarly examined. As shown in Figure 3B,

immunoprecipitation of GCN5 resulted in the recovery of nuclear RelA, particularly after TNF stimulation.

Next, the mechanism of binding between GCN5 and RelA was evaluated. Deletion mutants of RelA encompassing the RHD (amino acids 1-305), the transactivation domain (amino acids 306-551) or the COMMD1 interacting region (amino acids 1-180) were expressed in mammalian cells along with GCN5. RelA was subsequently precipitated from cellular lysates to detect the co-precipitation of GCN5. Unlike COMMD1 and SOCS1, which bind to the amino terminal end of the RHD (amino-acids 1-180) (Maine et al., 2007), GCN5 bound to the carboxy terminus of RelA (Figure 3C). Thereafter, we evaluated the elements within the carboxy terminal region of RelA that were required for GCN5 binding. In this case, mutants with progressive deletions from the carboxy terminus of RelA were expressed in mammalian cells and subsequently precipitated to detect the presence of endogenous GCN5. As shown in Figure 3D, a deletion of the last 21 amino acids of RelA, a highly conserved serine-rich area of the protein, was sufficient to greatly diminish binding, indicating that this region contains the main binding interface between these two proteins. Similar binding studies were also performed utilizing bacterially-made RelA fragments and *in vitro* translated GCN5, giving identical results (data not shown).

The last 21 amino acids of RelA include serine 536, which is an IKK phosphorylation site (Perkins, 2006) and has been previously implicated in the degradation of RelA (Lawrence et al., 2005). Therefore we examined whether serine 536 was required for binding between RelA and GCN5. When expressed in cells, a S536A point mutant of RelA was able to precipitate endogenous GCN5 without any appreciable difference to wild-type RelA (Figure S1, Available

online). This was in contrast to the effect of the deletion of the last 21 amino acids, indicating that other elements within this region and not serine 536 alone are required for binding to GCN5.

GCN5 promotes RelA ubiquitination

The ability of GCN5 to bind to COMMD1 and its inhibitory effect on NF-κB suggested that similar to COMMD1, GCN5 might promote RelA ubiquitination. To examine this notion, cells were transfected with HA-tagged RelA and His_6-tagged Ubiquitin, which was subsequently precipitated from cellular lysates with Nickel agarose beads to recover ubiquitinated proteins. Among the recovered material, the presence of ubiquitinated RelA was determined by immunoblotting. GCN5 expression promoted greater recovery of RelA in the ubiquitinated fraction, which appeared as laddered material of increasingly greater molecular weight, consistent with polyubiquitinated RelA (Figure 4A, left). Conversely, decreased GCN5 expression as a result of siRNA transfection led to decreased recovery of ubiquitinated RelA under basal conditions (Figure 4A, right). Importantly, to be able to conclude that the effect of GCN5 is on the rate of RelA ubiquitination *in vivo*, the experiment was designed to maintain equal input levels of RelA (third panel) and to achieve equal recovery of total ubiquitinated material among the samples (second panel).

Next, we examined whether GCN5 could promote the ubiquitination of endogenous RelA. In these experiments, endogenous RelA was precipitated from cellular lysates after protein denaturation and the material recovered was subsequently immunoblotted with an antibody that recognizes polyubiquitin chains. As shown in Figure 4B, the amount of immunoprecipitated RelA was identical across all groups, but the ubiquitinated material recovered was increased after short blockade of the proteasome with MG-132, particularly in

response to GCN5 expression. Altogether, these results indicated that similar to COMMD1, GCN5 promotes the ubiquitination of RelA.

GCN5 interacts with other components of the COMMD1-containing ligase

Since GCN5 can promote RelA ubiquitination, we speculated that GCN5 might interact with other COMMD1-associated factors involved in RelA ubiquitination. To examine this possibility, we first evaluated whether GCN5 containing complexes interact with a ubiquitin ligase in a functional assay. To that end, GCN5 fused with the affinity tag GST was expressed in mammalian cells and subsequently precipitated from cell lysates. The recovered complexes were offered as a potential source for ubiquitin ligase activity to an *in vitro* ubiquitination reaction containing the E1 and E2 (UbcH5a) enzymes, recombinant HA-tagged ubiquitin and an ATP regenerating buffer. Polyubiquitin chain formation was readily detectable when GCN5 precipitated from mammalian cells was provided as a source of ubiquitin ligase activity (Figure 4C). On the other hand, recombinant GST or GCN5-GST proteins prepared in *E. coli* were devoid of ubiquitin ligase activity in this assay (note that recombinant GCN5-GST could readily bind to RelA as shown in Figure 3A). This latter finding suggested that the ligase activity recovered from GCN5 expressed in mammalian cells was likely secondary to its association with other cellular factors and not intrinsic to the protein.

Given the above results, we examined in co-precipitation experiments whether GCN5 could interact with Cul2, the main scaffold subunit of the COMMD1-containing ligase. First, we precipitated GST-GCN5 from cell lysates, and consistent with our hypothesis, this material also co-precipitated endogenous Cul2 (Figure 4D). Interestingly, the recovered Cul2 was primarily in the neddylated form (the upper immunoreactive band), which represents the more active pool of

the enzyme and is also the primary form of the protein found in the cell nucleus where GCN5 resides (Maine et al., 2007). Additionally, while we previously reported that the Cul2-COMMD1 interaction is inducible by TNF (Maine et al., 2007), GCN5 associates with Cul2 in a manner that is not affected by TNF stimulation (Figure S2). Consistent with the notion that GCN5 not only interacts with ECS components but it is functionally linked to this complex, we observed that GCN5 deficiency impaired the ability of COMMD1 to promote RelA ubiquitination (Figure S3).

GCN5 is best known as a transcriptional activator, and it is associated with various large multimeric complexes in mammalian cells, such as the TFTC and STAGA complexes, which are characterized by the presence of several TAF proteins (Thomas and Chiang, 2006). Hence, we examined next whether the pool of GCN5 that associates with Cul2 could be concurrently interacting with either one of this complexes. To this end, we performed a large scale affinity purification of Cul2-containing complexes from mammalian cells and examined the recovered material for the presence of GCN5 and TAF6, a common component of both the TFTC and STAGA complexes. Cul2 fused with the affinity tag GST was expressed and was subsequently precipitated from cell lysates through a glutathione sepharose column; this material was extensively washed and Cul2 containing complexes were then eluted. As expected, Cul2-containing complexes were enzymatically active (data not shown) and contained expected partner proteins such as COMMD1, Elongin C and Rbx1 (Figure 4E, top 4 panels). Importantly, the same material also contained GCN5, but not TAF6 (Figure 4E, bottom 2 panels), suggesting that GCN5 binding to Cul2 does not concurrently involve the TFTC or STAGA complexes.

The data presented thus far indicated that the ubiquitin ligase activity associated with GCN5 is likely the results of its interaction with Cul2. However, it was recently reported that

PCAF, a close homolog of GCN5, possesses intrinsic ubiquitin ligase activity in its amino-terminus, a region that is highly conserved between the two proteins (Linares et al., 2007). To examine the possibility of a similar activity in the amino-terminus of GCN5, we precipitated GCN5 full-length or two truncation mutants from cell lysates (designated here N-term and HAT/Bromo, and spanning amino acids 1-491 and 491-837, respectively). GCN5 full-length was able to robustly induce polyubiquitin chain formation *in vitro*; interestingly, the carboxy-terminus of the protein and not the amino-terminus was able to maintain this activity (Figure 4F). In fact, additional experiments indicated that the carboxy-terminus of GCN5 is the region responsible for its interaction with COMMD1 and Cul2 (Figure 4G), while the amino terminus of the protein is responsible for its interaction with RelA (Figure S4). Hence the recovery of ubiquitin ligase activity was consistent with the co-precipitation of a Cul2-containing complex, and supports the notion that GCN5 promotes RelA ubiquitination through its interaction with the ECS^{SOCS1} complex.

GCN5 binding to RelA is enhanced by IKK-mediated phosphorylation

The data indicated that GCN5 could bind to the carboxy terminus of RelA, particularly to the last 21 amino acids, a serine-rich region where at least one IKK-phosphorylation site has been identified. Therefore, we decided to investigate if the effects of GCN5 and IKK on the ubiquitination of RelA could be functionally linked. To that end, we examined first if phosphorylation of RelA could promote its interaction with GCN5. Cells were transfected with GCN5 fused with GST, and were subsequently treated with stimuli that activate the IKK complex, namely TNF, IL1-β and Flagellin. GCN5 was precipitated from cell lysates and the presence of co-precipitated endogenous RelA was assessed by immunoblotting. In each case,

cell stimulation resulted in increased recovery of RelA (Figure 5A), and stimulation resulted in the expected transient degradation of IκB-α, an indirect marker of IKK activation (data not shown). In addition, Calyculin A was utilized to induce the accumulation of phosphorylated RelA, which resulted in a significant enhancement of its binding to GCN5 (Figure S5).

One potential explanation for the above results could be that all these stimuli increase the levels of RelA in the nucleus, where GCN5 is found, and might not be affecting the affinity between RelA and GCN5. To discriminate between these two possibilities, we evaluated the binding between GCN5 and RelA *ex vivo* (Figure 5B). Cell lysates were prepared after TNF or Calyculin A treatment and offered to recombinant GCN5-GST protein immobilized onto GSH beads. After precipitation, endogenous RelA could be easily detected and treatment of the cells with either stimulus enhanced the ability of GCN5 to bind to RelA. This effect was most significant with Calyculin A treatment, which causes more dramatic accumulation of phosphorylated RelA than TNF. Since the interaction between GCN5 and RelA in this experiment occurred post-lysis, the enhanced co-precipitation could not be explained by cellular redistribution of RelA.

In complementary experiments, recombinant GST-RelA could precipitate endogenous GCN5, but the amount recovered was not affected by Calyculin A treatment (data not shown), contrary to the observed enhancement of binding when recombinant GCN5-GST was used to precipitate endogenous RelA (Figure 5B). This supported the notion that a phosphorylation event on RelA might be responsible for its increased binding to GCN5. To test this possibility further, we treated the cellular lysates with recombinant λ-protein phoshatase (λ-PPase) to dephosphorylate RelA in the lysate. Phosphatase treatment of the lysate completely abrogated the increased binding between GCN5 and RelA, and returned it to the levels of the unstimulated

sample (Figure 5C, top panel). In addition, phosphorylated RelA was found to bind to GCN5 after Calyculin A treatment, but not after phosphatase treatment of the lysate (Figure 5C, second panel). As expected, λ-PPase resulted in the dephosphorylation of RelA, best seen by the changes in electrophoretic mobility of RelA before and after phosphatase treatment (Figure 5C, bottom two panels). These experiments were consitent with the notion that phosphorylation of RelA enhances its binding to GCN5.

Next, we sought to identify whether the IKK complex, which is required for the degradation of RelA upon phosphorylation (Figure 1C), might be also involved in the inducible binding between RelA and GCN5. To test this possibility, wild-type, or *Ikkα* or *β*-deficient MEFs were treated with TNF and cellular lysates were subsequently mixed with recombinant GCN5 protein. As before, TNF greatly enhanced the binding between GCN5 and RelA, but this was observed only when using lysates from wild-type cells, and not from *Ikk*-deficient cells (Figure 5D).

Prior data indicated that the IKK complex is involved in phosphorylation-dependent ubiquitination and degradation of RelA (Figure 1) and is additionally required to induce enhanced binding between GCN5 and RelA (Figure 5D). Therefore, we predicted that GCN5 would be required for the degradation of RelA in response to phosphorylation. To test this notion, we examined whether decreased GCN5 expression could impair Calyculin-mediated degradation of RelA, in a manner akin to the effect of *Ikk* deficiency. Wild-type MEFs were infected with lentiviruses expressing short hairpin RNA sequences targeting murine Gcn5, and two independent targeting sequences resulted in 34% and 35% mRNA expression compared to control cells. Calyculin A treatment induced the degradation of RelA in the control group, but Gcn5-deficient cells were more resistant to the effects of Calyculin A (Figure 5E), akin to the

observations using *Ikk*-deficient fibroblasts (Figure 1C). Altogether, these results indicate that the IKK complex is required for RelA phosphorylation events that trigger its binding to GCN5.

The HAT activity of GCN5 is not required to induce RelA degradation

RelA has been previously reported to be acetylated in response to NF-κB activation by at least two enzymes, p300 and PCAF (Perkins, 2006). Given the close homology between PCAF and GCN5, and the fact that the HAT activity of GCN5 has been implicated in all of its cellular functions to date, we examined whether the HAT activity of GCN5 might participate in RelA ubiquitination.

First, we examined if RelA could be acetylated by GCN5. RelA was expressed in cells (fused to GST), along with p300, PCAF or GCN5 and was subsequently precipitated from cellular lysates. As shown in Figure S6A, the amount of precipitated RelA was comparable across all groups (middle panel), but only the expression of p300 was able to promote significant accumulation of acetylated RelA (upper panel). Therefore, at least under the constraints of our detection systems, there was no evidence of GCN5-mediated acetylation of RelA, while p300-mediated acetylation was readily detectable.

This result does not rule out the involvement of the HAT activity of GCN5 in the ubiquitination of RelA, perhaps acting on a different substrate such as a component of the ECS^{SOCS1} complex. To examine this possibility, we introduced a point mutation in the HAT domain of GCN5 that has been previously shown to inactive the catalytic core of the enzyme (Tanner et al., 1999). Consistent with the prior report, we observed that the point mutant E575Q was unable to self-acetylate, a testament to the expected enzymatic inactivation (Figure S6B). In addition, GCN5 possesses a highly conserved Bromo domain, a region present in various HATs

that is capable of interacting with acetylated lysine residues. Therefore, we similarly introduced a Y814A point mutation that is known to disrupt acetylated-lysine recognition (Hudson et al., 2000).

The ability of these point mutants to promote RelA ubiquitination was examined next, utilizing a similar experimental approach as that described in Figure 4A. As can be appreciated in Figure 6A, expression of wild-type GCN5, or either of the point mutants was capable of promoting RelA ubiquitination. Therefore, neither the HAT activity nor the Bromo domain are required for GCN5-promoted ubiquitination of RelA.

We previously reported that COMMD1 mediated ubiquitination leads to the degradation of RelA, and hence we examined if either wild-type or the HAT deficient point mutant E575Q could promote the degradation of RelA. As shown in Figure 6B, expression of either form of GCN5 led to decreased protein levels of RelA, and this effect was synergistic when COMMD1 or Cul2 were co-expressed with GCN5. This effect was not due to a decrease in RelA expression at the mRNA level (data not shown). Finally, consistent with the ability of GCN5 to promote RelA degradation, the wild-type protein or the E575Q mutant were equivalent in their inhibition of RelA-mediated *NFKBIA* expression at the mRNA levels (Figure 6C, upper bar graph) or the protein level (lower panel, IκB-α western blot). Again, the inhibitory effects of GCN5 were synergized by concurrent expression of COMMD1 or Cul2.

If the HAT and bromo domains of GCN5 are not involved in the RelA ubiquitination, it would be predicted that the point mutations in these domains should not affect GCN5 interactions with RelA or with component of the ligase, such as COMMD1 or Cul2. Indeed, the ability of RelA to co-precipitate GCN5 was not affected by the E575Q or the Y814A mutations (Figure S7A), and similarly, these mutants demonstrated no impairment in their ability to interact

with COMMD1 or Cul2 (Figure S7B). Altogether these results indicate that GCN5-promoted RelA ubiquitination is separable from its known HAT activity or the function of its Bromo domain, establishing this effect as a previously unrecognized biochemical property of GCN5.

DISCUSSION

The regulation of NF-κB mediated transcription relies greatly on the IKK-IκB axis, which primarily controls DNA binding and nuclear accumulation of the transcription factor (Karin et al., 2004). However, it is also clear that a series of steps need to take place in order for nuclear NF-κB dimers to induce transcription. Homodimers of the p50 subunit, which repress transcription, are exchanged for heterodimers containing RelA, c-Rel or RelB proteins, in a process that requires the ubiquitination and proteasomal degradation of p50 homodimers (Carmody et al., 2007; Zhong et al., 2002). In addition, a series of specific post-translational modifications of NF-κB subunits such as phosphorylation and acetylation are necessary for their interaction with co-activator complexes, ultimately triggering transcriptional activation.

An equally important phase in the regulation of NF-κB activity is the termination of transcription. This phase of the response has been largely ascribed to NF-κB mediated re-synthesis of IκB proteins, which subsequently mediate NF-κB nuclear export, in a classical negative feedback loop (Hoffmann et al., 2002). In this context, ubiquitination of NF-κB has been found to be an additional mechanism of transcriptional termination (Saccani et al., 2004). At least one of the ligases responsible for this effect contains COMMD1 in association with is a multimeric complex between Elongins B/C, Cul2, and SOCS1, termed the ECS^{SOCS1} complex (Maine et al., 2007; Ryo et al., 2003).

Prior work identified that IKKα-mediated phosphorylation of RelA, a serine protein kinase, was an important regulator of protein stability and was required for transcriptional termination (Lawrence et al., 2005). While serine phosphorylation events can be recognized by $SCF^{\beta TrCP}$, a Cul1-containing ubiquitin ligase, the ECS^{SOCS1} complex which contains Cul2, is not

known to engage phospho-serine substrates (Petroski and Deshaies, 2005). In fact, SOCS1-promoted ubiquitination of other proteins, such as JAK2, is dependent on the interaction between phospho-tyrosine residues in the target protein and the SH2 domain of SOCS1 (Kamizono et al., 2001). Therefore, it seems unlikely that a serine phosphorylation event on RelA could have an impact on its interaction with SOCS1. Furthermore, prior studies indicated that RelA binding to the ECS^{SOCS1} complex is mediated by the cooperative interaction of COMMD1 and SOCS1 with the amino-terminus of the RelA (Maine et al., 2007), a region where IKK-mediated phosphorylation events have not been described to date (Perkins, 2006). Hence, a connection between IKK phosphorylation of RelA and its subsequent ubiquitination was not evident prior to this study.

Our findings indicate that the link between serine phosphorylation and ubiquitination is mediated by GCN5, which can work as an accessory subunit to the COMMD1-containing ligase. GCN5 was identified first among COMMD1- associated factors, and was similarly found to be present in Cul2-containing complexes. Furthermore, our studies demonstrate that GCN5 is required for RelA ubiquitination and degradation, particularly in response to IKK-mediated phosphorylation. The identification of GCN5 in this role indicates that this ligase not only contains its known core components (Elongins B and C, Cul2, SOCS1 and Rbx1), but also additional accessory factors. This notion is consistent with previous reports of accessory factors for other Cullin-containing ubiquitin ligases (Ganoth et al., 2001), and the fact that the APC/Cyclosome, a multimeric ligase containing the Cullin homolog APC2, is known to have great subunit complexity (Thornton et al., 2006).

An intriguing finding of our study is the identification of a role for GCN5 in protein ubiquitination, which our data indicate is separable from its HAT activity. Genetic evidence

indeed suggests that GCN5 provides functions that are HAT-independent. Deficiency of *Gcn5* in mice prevents the formation of somites, a neural tube or a notochord soon after gastrulation (Bu et al., 2007), while a HAT inactivating mutation in the *Gcn5* gene results in a milder phenotype where embryos progress until mid-gestation (E11.5), but exhibit severe cranial tube defects (Bu et al., 2007). Similarly, PCAF, a close homolog of GCN5 present in vertebrates, has been recently implicated in the ubiquitination of Mdm2, and it was postulated that it functions through an intrinsic ubiquitin ligase activity provided by its amino terminus (Linares et al., 2007). Intriguingly, despite the significant homology between PCAF and GCN5, our findings indicate that GCN5-associated ligase activity maps to its carboxy terminus, the same area that mediates its interaction with Cul2. In addition, bacterially-made recombinant GCN5 failed to have ligase activity *in vitro* despite the fact that the same protein retained its ability to bind to RelA. These findings demonstrate that despite the similarities between GNC5 and PCAF, and their involvement in protein ubiquitination, the mechanisms by which they promote ubiquitination of targets could be quite dissimilar.

Phosphorylation-dependent degradation of RelA requires the IKK complex and GCN5 (Figures 1C and 5E). Consistent with a functional relationship between these factors, IKK dependent phosphorylation of RelA promotes its binding to GCN5. In particular, the last 21 amino acids of RelA are critical for its binding to GCN5 under basal and stimulated conditions. However, our studies indicate that a phosphorylation site other than serine 536 or the involvement of multiple phosphorylation events, probably account for the inducible binding between RelA and GCN5. Finally, these findings provide a mechanism to link IKK activation, RelA phosphorylation, and the degradation of the protein. Hence, the activation of the IKK complex, not only initiates NF-κB mediated transcription, but also sets in motion a process that

ultimately promotes the termination of transcription through the ubiquitination and degradation of the transcription factor in a manner that depends on GCN5.

EXPERIMENTAL PROCEDURES

In vitro binding experiments. GST fusion proteins bound to GSH sepharose were incubated at room temperature with BSA (1 mg/mL) in 200μL of Z-buffer (25 mM HEPES, pH 7.5, 12.5 mM MgCl2, 150 mM KCl, 0.1% NP-40, 20% glycerol). After 15 min, *in vitro*-translated proteins were added and incubated for another 45 min at room temperature. The beads were then washed four times with 1 mL NETN buffer (100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 20 mM Tris-HCl, pH 8.0). Bound proteins were resolved by SDS-PAGE and subjected to immunoblotting or autoradiography, depending on the experiment. Protein dephosphorylation was performed by incubating cell lysates with λ-PPase (NEB) at 30°C for 30 minutes after addition of the recommended reaction buffer and Mn.

In vitro ubiquitination assays. Each reaction mixture consisted of recombinant ubiquitin (2.5μg), E1 (Uba1, 50ng), E2 (UbcH5a, 100ng), and ATP regenerating buffer (all obtained from Boston Biochem). These were mixed in reaction buffer (40mM HEPES pH 7.9, 60mM potassium acetate, 2mM DTT, 5mM MgCl2, 10% glycerol) and incubated at 30˚C for 90 minutes (Maine et al., 2007).

SUPPLEMENTAL DATA

Supplemental data include Supplemental experimental procedures, Supplemental references and seven Supplemental figures.

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FIGURE LEGENDS

Figure 1. IKK-mediated phosphorylation results in ubiquitination and degradation of RelA. (A) Phosphorylation promotes RelA degradation. Wild-type MEFs were incubated with Calyculin A (Calyc) for variable amounts of time and subsequently lysed in Triton X-100 buffer. RelA degradation and phosphorylation (at serine 536) were determined by immunoblotting. **(B) Phosphorylation promotes RelA ubiquitination.** MEFs were treated with MG-132 (30 min) and Calyculin A as indicated. RelA was subsequently immunoprecipitated from cell lysates and the presence of ubiquitinated RelA was determined by immunoblotting for ubiquitin. **(C) IKK is required for phosphorylation promoted RelA degradation.** Wild-type, *Ikkα* $^{-/-}$, or *Ikkβ* $^{-/-}$ MEFs were treated with Calyculin A for 30 minutes. Subsequently, cell lysates were prepared and the degradation and phosphorylation of RelA were determined by western blot analysis.

Figure 2. GCN5 binds to COMMD1 and inhibits NF-κB mediated transcription.
(A) Identification of GCN5 as a COMMD1-associated factor. A carboxy-terminal peptide of GCN5 (amino acids 817-828) was identified by tandem mass spectrometry after chromatography column purification of COMMD1-TAP (Burstein et al., 2005). Schematic representation of GCN5, with the boundaries of its histone acetyltransferase domain (HAT) and bromo domain (BD) is shown. The HAT activity and bromo domain function are dependent on residues E575 and Y814, respectively. **(B) COMMD1 binds GCN5 through its COMM domain.** GCN5 was co-expressed with full-length COMMD1 (FL), its amino-terminus (N), or its COMM domain (CD) in fusion with GST. Subsequently, COMMD1 was precipitated by GSH sepharose beads and the recovered material was immunoblotted for GCN5. **(C) Co-precipitation of**

endogenous GCN5 and COMMD1. Nuclear extracts were prepared from unstimulated or TNF stimulated HEK293 cells. These were subjected to immnoprecipitation for COMMD1, followed by immunoblotting for GCN5. **(D) GCN5 inhibits RelA-mediated transcriptional activation.** Cells were co-transfected with the 2κB-luciferase reporter, RelA, COMMD1, GCN5 or vector as indicated. Activation of NF-κB dependent transcription was determined by luciferase assay. **(E to I) GCN5 deficiency enhances endogenous NF-κB dependent gene expression.** HEK293 cells were transiently transfected with siRNA against GCN5 and stimulated with TNF, as indicated. Transcript levels for *TNF* (E) and *CX3CL1* (F) were measured by quantitative real-time RT-PCR (qRT-PCR). U2OS cells were transduced with a lentivirus expressing shRNA against GCN5 and stimulated with TNF, as indicated. Inhibition of GCN5 expression was demonstrated by western blot and qRT-PCR (G) and transcript levels for *ICAM1* (H) and *IL8* (I) were determined by qRT-PCR.

Figure 3. GCN5 interacts with the extreme carboxy-terminus of RelA. (A) RelA and GCN5 bind *in vitro*. Recombinant GST or GCN5-GST were adsorbed onto GSH sepharose beads and mixed with radiolabeled *in vitro* translated RelA. After precipitation, the material was resolved by SDS-PAGE and subjected to autoradiography (left panel) or coomasie staining (right panel). **(B) Co-precipitation of endogenous RelA and GCN5.** Nuclear extracts were prepared from unstimulated and TNF-stimulated HEK293 cells. GCN5 was immunoprecipitated from the nuclear extracts and the recovered material was immnoblotted for RelA. **(C) GCN5 interacts with the transactivation domain of RelA.** GCN5 was co-transfected with RelA full-length (FL), or truncation mutants spanning the indicated amino acids, fused to GST. Subsequently, GST proteins were precipitated from cell lysates and the recovered material was immunoblotted

for GCN5. **(B) The last 21 amino acids of the RelA are required for its interaction with GCN5.** Cells were transfected with RelA FL, or the indicated truncation mutants fused to GST. Subsequently, RelA was precipitated from cell lysates and immunoblotting was performed to detect endogenous GCN5.

Figure 4. GCN5 promotes RelA ubiquitination and interacts with the ECS^{SOCS1} complex. **(A) GCN5 promotes RelA ubiquitination.** Cells were transfected with HA-RelA and His_6-tagged ubiquitin, along with a GCN5 expression plasmid (left panel) or siRNA against GCN5 (right panel). Cells were lysed in 8M Urea lysis buffer and ubiquitinated proteins were precipitated by nickel beads. The presence of ubiquitinated RelA in the recovered material was determined by immunobloting (HA). **(B) GCN5 expression promotes the ubiquitination of endogenous RelA.** Cells were transfected with GCN5 and were treated with MG-132 (3 hours) as indicated. A denatured immnoprecipitation for RelA was subsequently performed from cell lysates and the presence of ubiquitinated RelA was determined by immunoblotting for ubiquitin. **(C) GCN5 precipitates ubiquitin ligase activity.** GCN5-GST precipitated from transfected HEK293 cells, or recombinant GST or GCN5-GST prepared in *E. coli*, were added to an *in vitro* ubiquitination reaction as a potential source for E3 ligase activity. Polyubiquitin chain formation during the reaction was determined by subjecting the sample to SDS-PAGE and immunoblotting for ubiquitin. **(D) GCN5 binds to Cul2, the main scaffold protein of the COMMD1-containing ligase.** Cells were transfected with GST or GST-GCN5, which were precipitated from cell lysates by GSH sepharose beads. The presence of co-precipitated endogenous Cul2 was determined by western blot analysis. **(E) GCN5 was co-purified as a Cul2-associated factor.** Lysates were prepared from HEK293 cells overexpressing a GST-Cul2 fusion protein (Input),

and were subsequently applied to a GSH-sepharose affinity column. Cul2-containing complexes were subsequently eluted from the column with excess GSH and concentrated by filtration (Eluate). The input, the flow-through material not bound to the column (FT) and eluate were subjected to immunoblotting for Cul2, COMMD1, Elongin C (EloC), Rbx1, GCN5 and TAF6. **(F) The carboxy-terminus of GCN5 containing the HAT and Bromo domains precipitates E3 ligase activity**. GCN5 full-length (FL) fused to GST or truncation mutants spanning its amino terminus (N-term, amino acids 1-491) or carboxy terminus (HAT/Bromo, amino acids 492-837) were expressed in mammalian cells. These proteins were precipitated from cell lysates, added to *in vitro* ubiquitination reactions, and polyubiquitin chain formation was determined as in (C). **(G) The carboxy terminus GCN5 binds to Cul2 and COMMD1.** Cells were transfected with GCN5 full-length (FL) fused to GST or the indicated truncation mutants along with Flag-Cul2 or COMMD1-Flag. GCN5 was precipitated by GSH sepharose beads and the presence of Cul2 or COMMD1 in the precipitated material was determined by western blot analysis.

Figure 5. IKK-mediated phosphorylation enhances GCN5-RelA interactions. (A) Various IKK-activating stimuli promote GCN5-RelA binding. Cells were transfected with GCN5-GST or GST and subsequently treated with TNF, IL-1β or Flagellin prior to lysis and GSH precipitation. The presence of co-precipitated endogenous RelA was determined by immunoblotting. **(B) TNF and Calyculin A treatment prior to lysis promotes RelA-GCN5 binding *ex vivo*.** HEK293 cells were treated with TNF or Calyculin A before lysis. Recombinant (r) GST or GCN5-GST were adsorbed onto GSH sepharose beads and mixed with the obtained lysates followed by precipitation. The presence of endogenous RelA in the

recovered material was determined by immunoblotting. **(C) Dephosphorylation of RelA abrogates its induced binding to GCN5.** HEK293 cells were treated with Calyculin A, and subsequently lysed in Triton X-100 lysis buffer without phosphatase inhibitors. This material was incubated with λ-protein phosphatase (λ-PPase) for dephosphorylation as indicated. Following the dephosphorylation reaction, the lysates were utilized for *ex vivo* binding of RelA to recombinant GCN5 as in (B). The recovered material was immunoblotted to detect the co-precipitation of endogenous RelA and phosphorylated RelA. The input lysates before and after λ-PPase treatment were immunoblotted for RelA. **(E) IKK is required for TNF- induced RelA-GCN5 binding.** Wild-type, *Ikkα* $^{-/-}$, or *Ikkβ* $^{-/-}$ MEFs were stimulated with TNF and subsequently lysed. This material was applied to recombinant GCN5-GST for *ex vivo* binding as described in (B). The recovered material was immunoblotted to detect the co-precipitation of endogenous RelA. **(E) Gcn5 is required for phosphorylation-promoted degradation of RelA**. Gcn5-deficient and control MEF cell lines were established using lentiviruses to deliver shRNA. These cells were treated with Calyculin A and subsequently lysed for western blot analysis to detect RelA degradation.

Figure 6. The HAT activity of GCN5 is not required for RelA ubiquitination and degradation. (A) GCN5 promotes RelA ubiquitination despite inactivating mutations in its HAT or Bromo domains. GCN5 wild-type, E575Q (HAT deficient), or Y814A (Bromo domain deficient) were co-transfected with HA-RelA and His_6-tagged ubiquitin. Ubiquitinated proteins were subsequently precipitated as in Figure 3A and the presence of ubiquitinated RelA was determined by immunoblotting (HA). **(B) GCN5 promotes the degradation of RelA independently of its HAT activity.** Cells were transfected with small amounts of RelA, along

with GCN5 wild-type or E575Q mutant, in combination with COMMD1 or Cul2 as indicated. Cells were lysed and the expression of RelA was determined by western blot. **(C) GCN5 inhibits RelA-mediated transcription independently of its HAT activity.** Cells were transfected as in (B) and RNA and protein were obtained and utilized to measure mRNA expression of the *NFKBIA* gene and the corresponding levels of the encoded IκB-α protein.

Figure 1
Mao *et al*

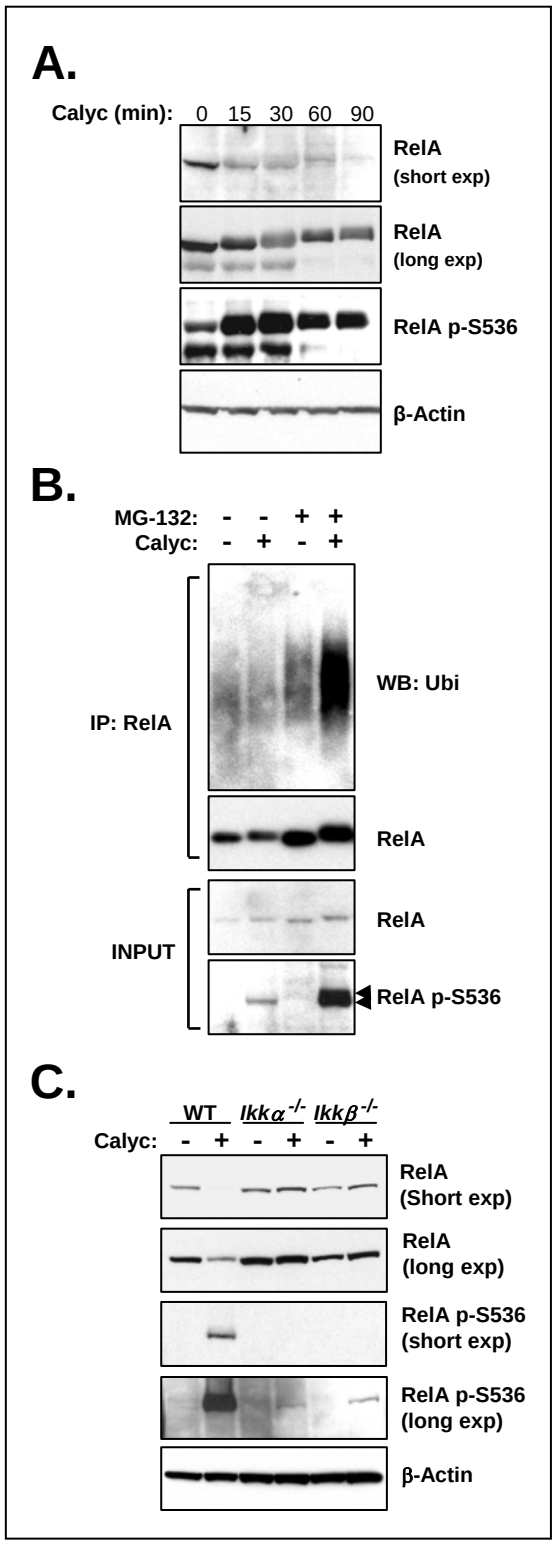

Figure 2
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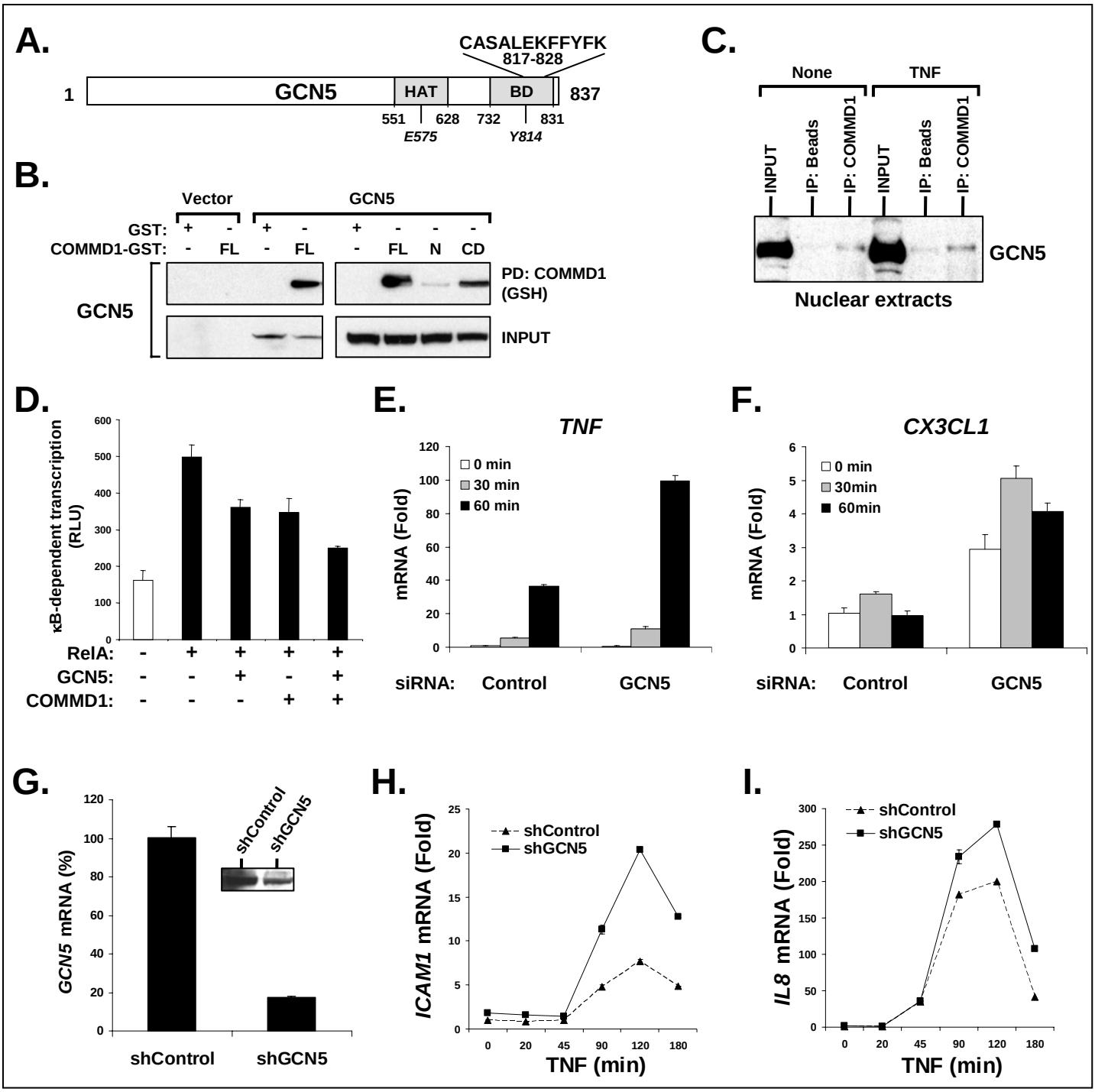

Figure 3
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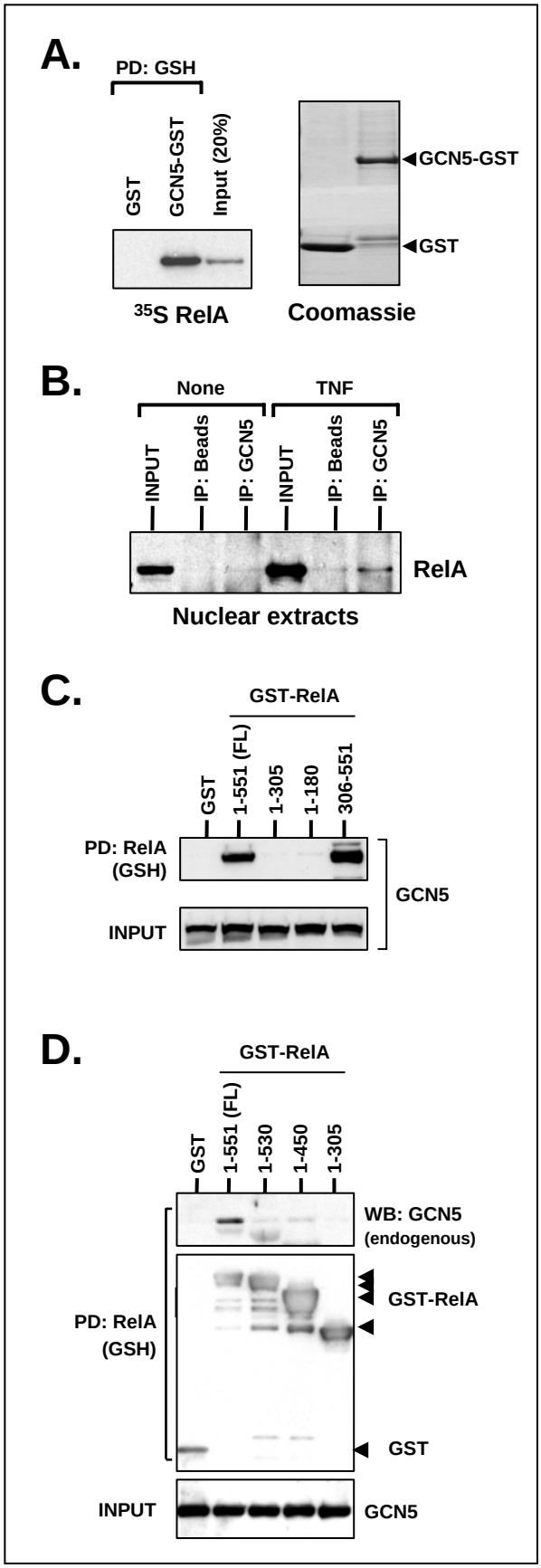

Figure 4

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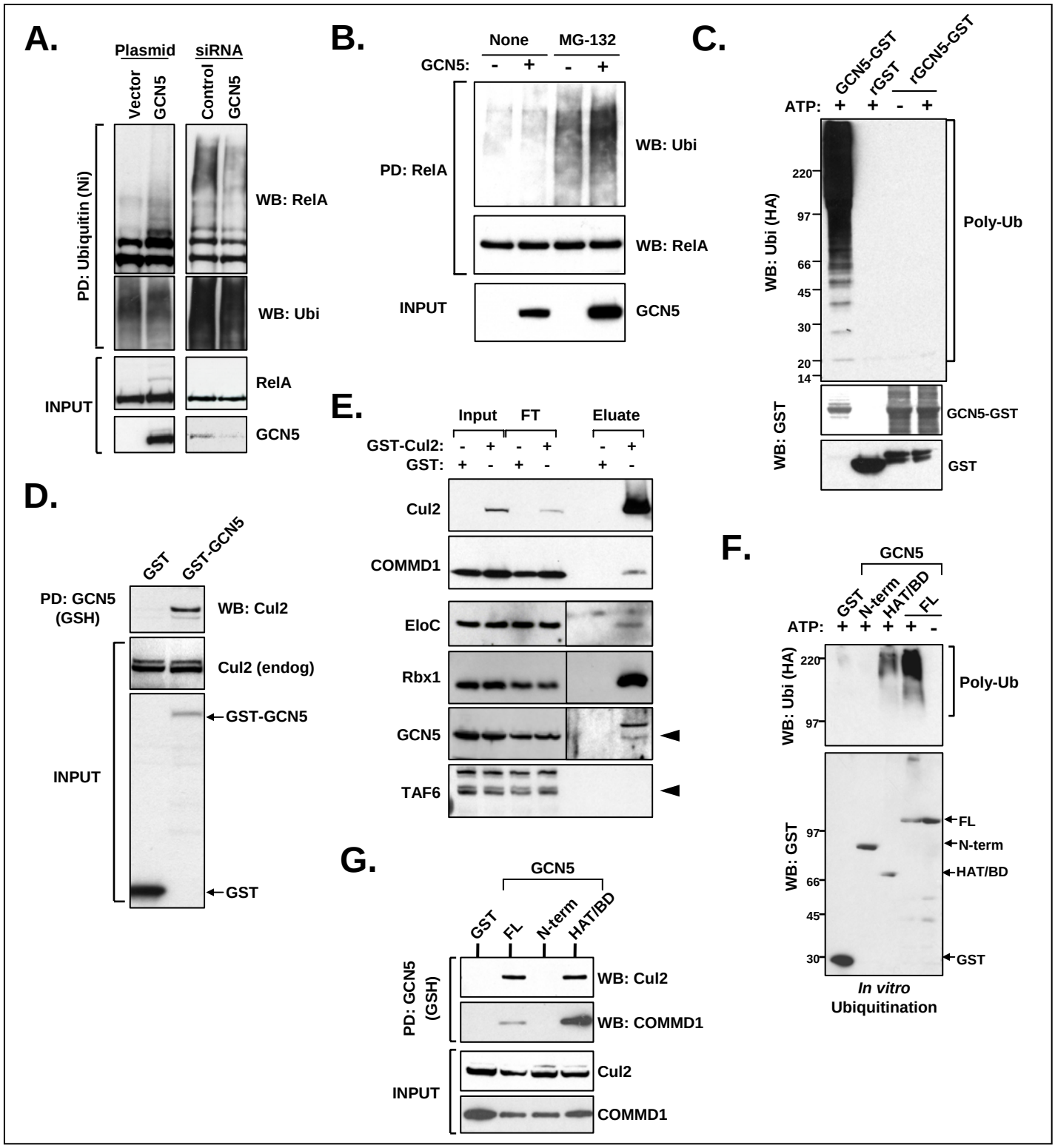


Figure 5

Mao *et al*

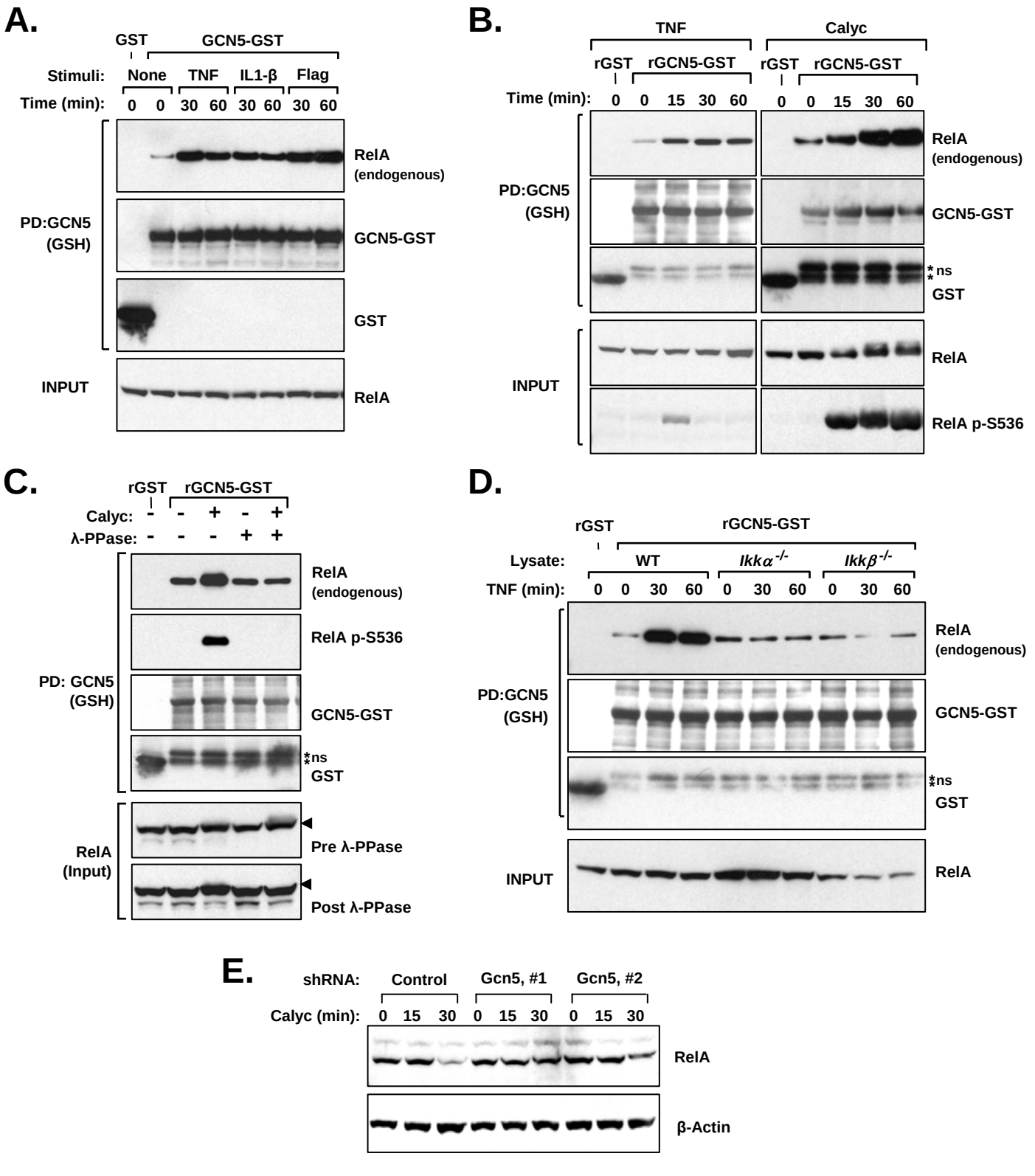
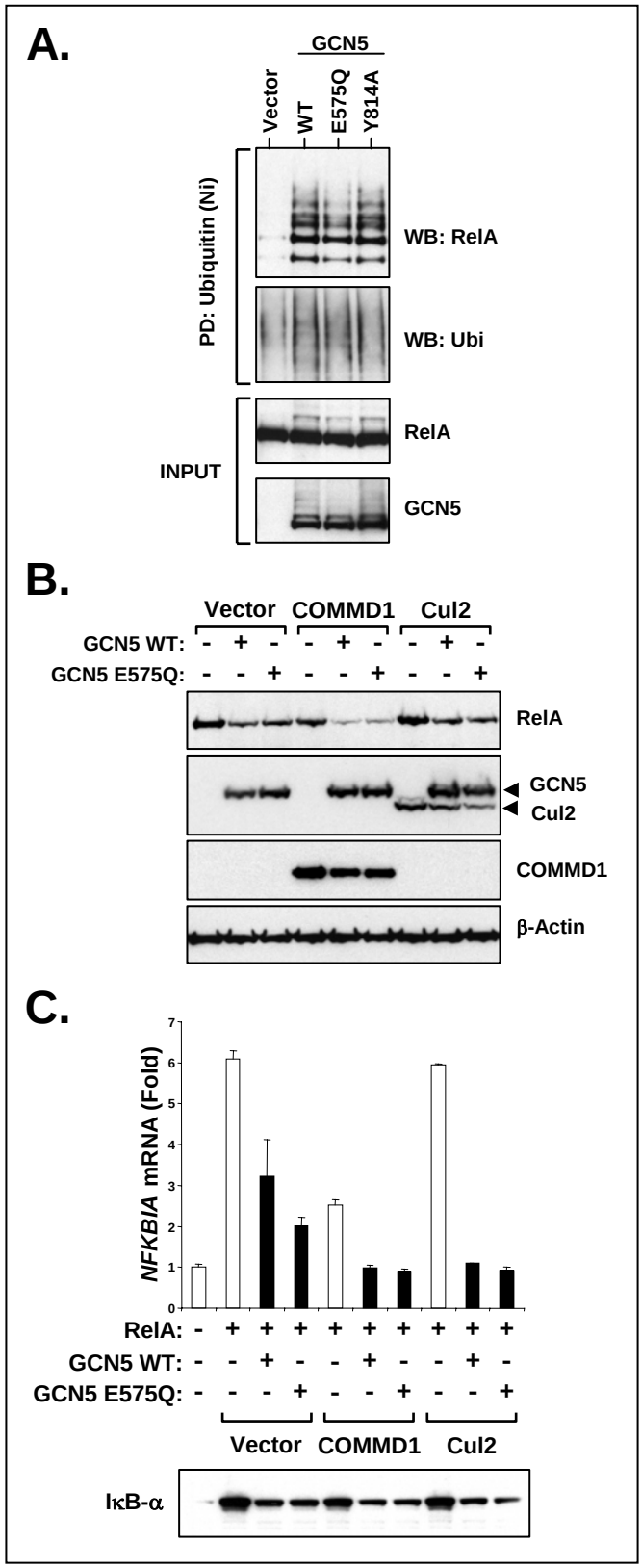


Figure 6

Mao *et al*



SUPPLEMENTAL DATA

Supplemental Experimental Procedures

Plasmids and small interfering RNA (siRNA). The plasmids pEBB, pEBG, pEBB-COMMD1-Flag, pEBB-COMMD1-GST FL, N-term (amino acids 1-118), COMM domain (amino acids 119-190), pEBB-Flag-Cul2, pEBG-Cul2 FL pEBG-RelA FL and its truncations 1-305, 1-180, 306-551, pEBB-HA-RelA, pCW7-His_6-Myc-Ubiquitin and 2κB-luc reporter have been described previously (Burstein et al., 2004; Burstein et al., 2005; Maine et al., 2007). The full length coding sequence for human GCN5 was obtained from an EST clone (IMAGE clone 5575574) to generate pEBB-GCN5 (untagged). Using this EST clone as template, PCR was utilized to generate the following plasmids: pcDNA3.1-GCN5, pEBB-Flag-GCN5, pET21b-GCN5-GST, pEBB-GCN5-GST and pEBG-GCN5 FL. Similarly, PCR was utilized to generate pEBG-GCN5 N-term (amino acid 1-491), pEBG-GCN5 HAT/Bromo domain (amino acid 492-837), pEBG RelA 1-530, pEBG RelA 1-450. pcDNA-Flag-p300 was a gift from Dr. Colin Duckett. Expression vectors for point mutants of GCN5 and RelA (pEBB-Flag GCN5 E575Q, Y814A and pEBG-RelA S536A) were created by site directed mutagenesis (Stratagene). Synthetic siRNA oligonucleotides against Chloramphenicol acetyl transferase (a control target), human GCN5, and mouse Gcn5 were utilized and detailed information about targeting sequences are available upon request. The plasmids pHCMV-VSV-G, pMDLg/pRRE, pRSV Rev and FG12 (Lois et al., 2002) were utilized for the generation of recombinant lentiviruses (kindly provided by Dr. David Baltimore). For stable RNAi utilizing lentiviral delivery, we introduced a cassette containing the

Histone 1 promoter and a short hairpin RNA from pSUPER, into the XbaI and XhoI sites of FG12.

Cell culture, transfection and luciferase assays. Human embryonic kidney (HEK) 293 cells, HEK 293T cells, and U2OS cells were obtained from ATCC and cultured in DMEM supplemented with 10% FBS and L-glutamine (2mM). *Ikk*$\alpha^{-/-}$, *Ikk*$\beta^{-/-}$ and wild-type mouse embryo fibroblasts were kindly provided by Dr. Inder Verma (Li et al., 1999a; Li et al., 1999b). All transient transfection experiments were performed in HEK293 cells using a standard calcium phosphate transfection protocol was used to transfect plasmids and siRNA (Burstein et al., 2004). In certain experiments, the following agents were applied at the indicated final concentration to the growth media: TNF (Roche, 1000U/mL), IL-1β (Roche, 10ng/mL), Flagellin (Inotek, 50μg/mL), Calyculin A (Cell Signaling, 50 nM), and MG-132 (Boston Biochem, 40 μM). Luciferase assays were performed as previously described (Burstein et al., 2005).

Quantitative RT-PCR. Total RNA was extracted from cells using the RNeasy kit (Qiagen) according to the manufacturer's instructions. An RT reaction with 500ng of total RNA in 25μL was performed using random hexamers and Taqman Reverse Transcription Reagents (Applied Biosystems). This was followed by quantitative PCR performed in the 7500 Real Time PCR system (Applied Biosytems). Oligonucleotides and internal probes for *NFKBIA, ICAM1*, *TNF* and *IL8* transcripts were obtained from Applied Biosystems, and a Taqman PCR Master Mix with *GAPDH* mRNA quantitation was duplexed in the same well as an internal control. To quantify *CX3CL1* mRNA, specific primers spanning over an intron were designed (sequences available upon request). These were combined with SYBERGreen PCR Master Mix for mRNA

quantitation and parallel reactions with primers targeting *GAPDH* were performed as a control for mRNA abundance.

Immunoblotting and immunoprecipitation. Whole cell lysates were prepared by adding Triton lysis buffer (25mM HEPES, 100mM NaCl, 10mM DTT, 1mM EDTA, 10% Glycerol, 1% Triton X-100), RIPA buffer (PBS, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS, 10mM DTT) or an 8M urea buffer (8M urea, 50mM Tris, pH 8.0, 300mM NaCl, 50 mM $NaPO_4$, 0.5% NP-40) supplemented with 1mM sodium orthovanadate and protease inhibitors (Roche), as indicated in each experiment. Preparation of cytosolic and nuclear extracts was performed as previously described (Burstein et al., 2005). Immunoprecipitations, GSH precipitations, Ni-NTA precipitations, and immunoblotting were performed as previously described (Burstein et al., 2004; Burstein et al., 2005). For denatured immunoprecipitation, cells were harvested and lysed initially in Triton X-100 Buffer (75μL). The lysate was then mixed with equal volume of TSD buffer (50mM Tris pH7.5, 2% SDS, 5mM DTT) and sonicated for 10 seconds. Subsequently, the lysate was heated at 80˚C for 5 min and Triton X-100 buffer was added to a final volume of 1,500 μL (or a final concentration of 0.1% SDS) prior to conventional immunoprecipitation. For *ex vivo* binding assays, *E. Coli* expressed GST fusion proteins were loaded onto glutathinone sepharose beads (Burstein et al., 2005) and 2-5μg of recombinant protein was mixed with mammalian cell lysates to co-precipitate its binding partner. The following antibodies were utilized in our studies: Acetylated lysine (Cell Signalling, 9441), β-Actin (Sigma, A5441), COMMD1 (Burstein et al., 2005), Cul2 (Zymed, 51-1800), Elongin C (BioLegend, 613101), Flag (Sigma, A8592), GST (Santa Cruz, sc-459), GCN5 (Santa Cruz, sc-20698, sc-6306), HA (CoVance, MMS101R), IκB-α (Upstate

Biotechnology, 06-494), IKKα (Cell Signaling, 2682), IKKβ (Cell Signaling, 2684), Rbx1 (LabVision, 127-075-160), RelA (Santa Cruz, sc-372 and sc-8008), RelA S-536 phospho (Cell Signaling, 3031), Ubiquitin (Stressgen, SPA-205). The TAF6 antibody was a kind gift from Dr. Robert Roeder.

Supplemental References

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Figure S1.

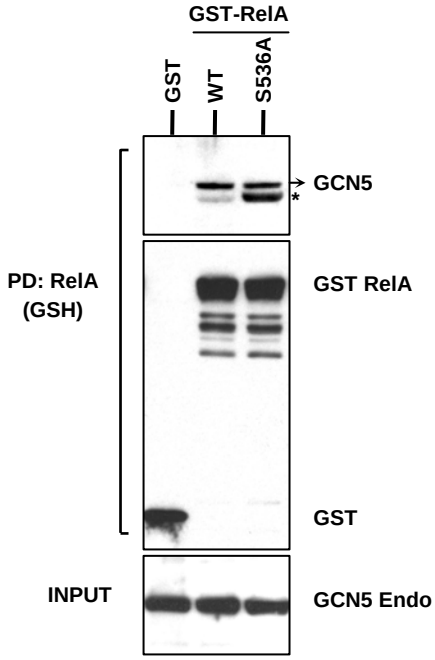

Figure S2. Mutation of S536A in RelA does not impair its interaction with GCN5. HEK293 cells were transfected wild-type and S536A mutant RelA. The cells were lysed and the lysates were utilized for GSH precipitation. The recovered material and the input were resolved by SDS-PAGE and immunoblotted for RelA and endogenous GCN5. The asterix indicates a non-specific band.

Figure S2.

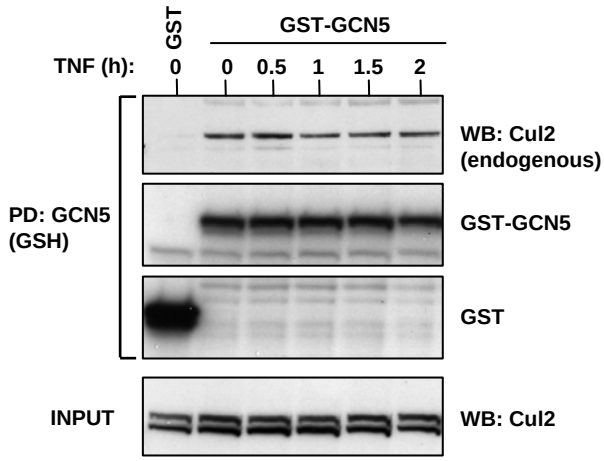

Figure S3. The Cul2-GCN5 interaction is not affected by TNF. HEK293 cells transfected with GCN5 fused to GST were treated with TNF for variable amounts of time as indicated. Subsequently, lysates were prepared from the cells and utilized for GSH precipitation. The precipitated materials were immnoblotted for GCN5 and endogenous Cul2.

Figure S3.

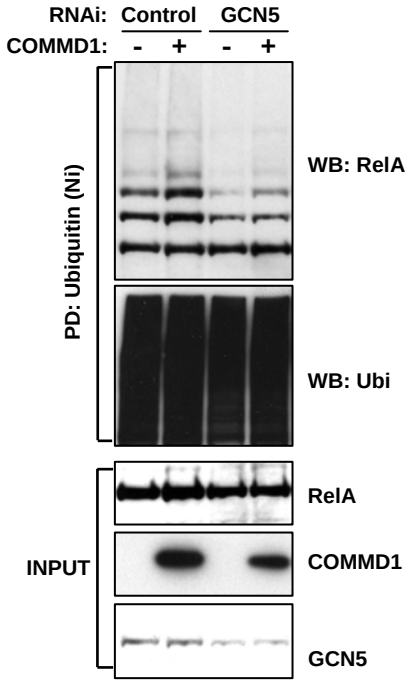

Figure S4. GCN5 deficiency impairs COMMD1 promoted RelA ubiquitination. HEK293 cells were transfected with HA-RelA and His_6-tagged ubiquitin, as well as COMMD1 as indicated. Endogenous GCN5 levels were suppressed by transfection of siRNA. Cells were subsequently lysed in 8M Urea lysis buffer and ubiquitinated proteins were precipitated by nickel beads. The recovered material was immunoblotted for RelA and polyubiqutin, and the input lysate was immunoblotted for RelA and GCN5.

Figure S4.

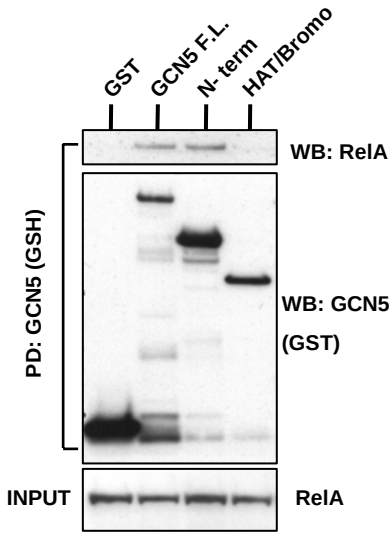

Figure S5. The amino-terminus of GCN5 binds to RelA. GCN5 full-length (FL) fused to GST or the indicated truncation mutants were expressed in HEK293 cells along with HA-RelA. GCN5 was precipitated by GSH sepharose beads and the recovered material was immnoblotted for RelA and GCN5.

Figure S5.

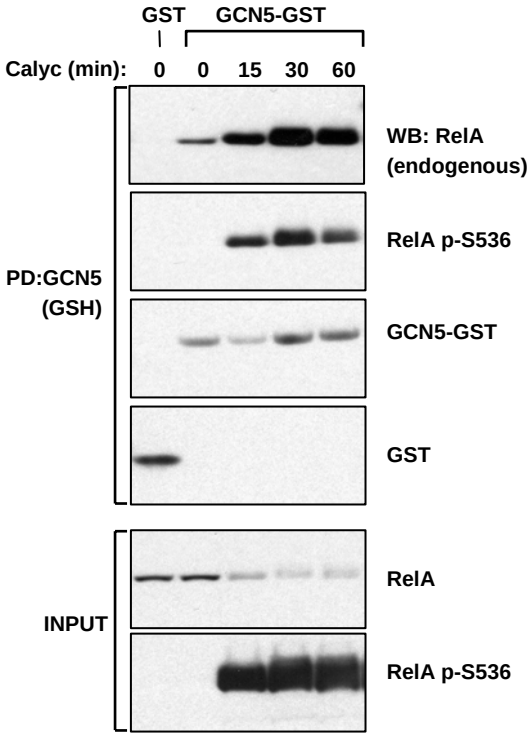

Figure S6. Calyculin A promotes the interaction between RelA and GCN5 *in vivo.* HEK293 cells transfected with GCN5 fused to GST were treated with Calyculin A for variable amounts of time. Subsequently the cells were lysed and GCN5 was precipitated with GSH sepharose beads. The recovered material was resolved by SDS-PAGE and immunoblotted for endogenous RelA, endogenous phosphorylated RelA and GST. Levels of RelA and phosphorylated RelA were determined in the input lysate by western blot analysis.

Figure S6.

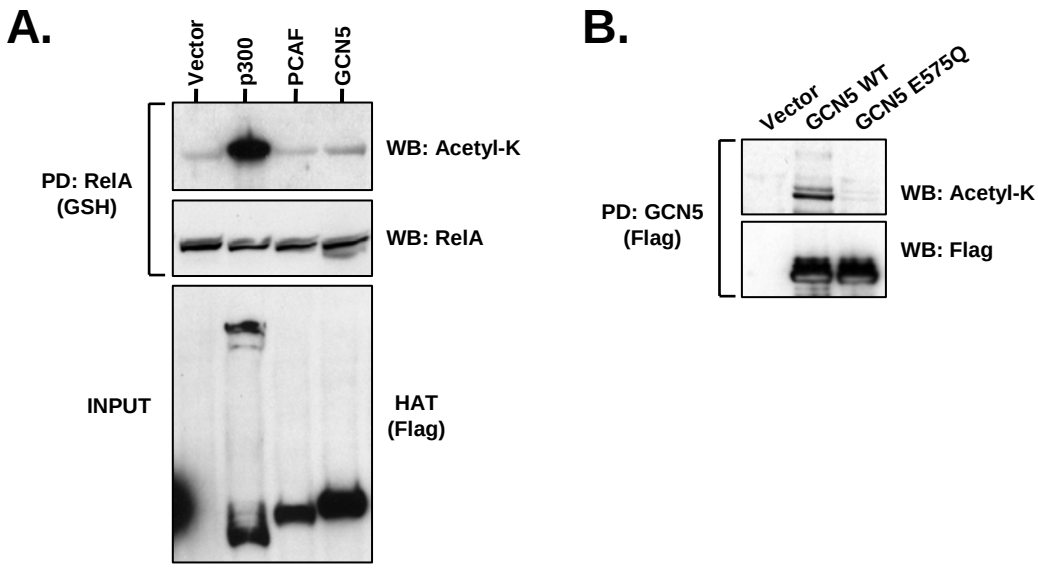

Figure S7. Acetylation is not involved in GCN5-meidated regulation of RelA. (A) GCN5 does not acetylate RelA. HEK293 cells were transfected with RelA fused to GST with Flag-p300, PCAF and GCN5. RelA was precipitated by GSH sepharose beads and subjected to immnoblotting for acetyled lysine (top panel) and RelA (middle panel). Expression of the transfected histone acetylases were determined by western blot analysis in the input lysate (bottom panel). **(B) GCN5 E575Q mutation abrogates its acetylation activity.** Wild-type GCN5 and E575Q mutant were expressed in HEK293 cells. The cells were lysed and utilized for denatured immnoprecipitation. The recovered material resolved by SDS-PAGE and immunoblotted for acetylated lysine to examine the self acetylation of GCN5 (top panel). The recovery of GCN5 was confirmed in both samples by immunoblotting (bottom panel).

Figure S7.

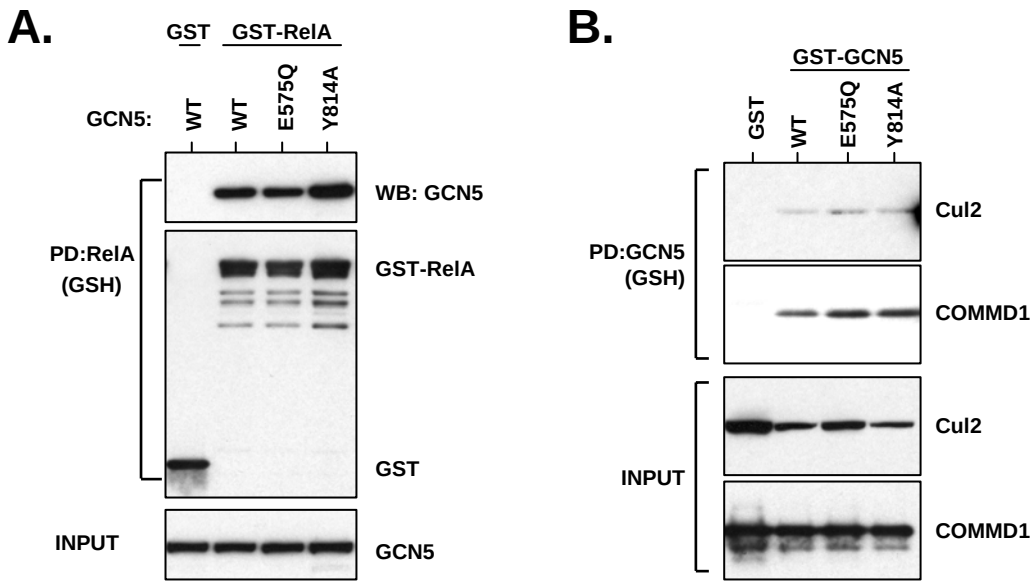

Figure S8. GCN5 E575Q and Y814A bind to RelA, Cul2 and COMMD1. **(A) GCN5 wild-type and its mutants E575Q and Y814A bind similarly to RelA.** RelA fused to GST was expressed in HEK293 cells along with wild-type Flag-GCN5 or its mutants E575Q and Y814A. The cells were lysed and subsequently subjected to GSH precipitation. Immunoblotting for GCN5 was performed on the precipitated material and the input lysate. GST immunoblotting was also performed to determine the amount of precipitated RelA. **(B) GCN5 E575Q and Y814A maintian their ability to bind to Cul2 and COMMD1.** Wild-type GCN5 or its mutants E575Q and Y814A fused to GST was expressed in HEK293 cells along with Flag-Cul2 or COMMD1-Flag. Subsequently, cell lysates were prepared and subjected to GSH precipitation, followed by immunoblotting for Cul2 and COMMD1 on the precipitated material and the input lysate.